An Analysis of Craniosynostotic Osteoprogenitor Cells and their Potential for Bone Tissue Engineering

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This thesis is dedicated to my parents Jean & David

for the loving home in which I was raised
for your unconditional support

❖

Abstract

The limited availability of autologous bone for grafting post-surgical defects in patients with craniosynostosis, necessitates the use of alloplastic materials which are prone to rejection or infection. The use of bio-composite materials consisting of alloplastic bioabsorbable scaffolds and autologous osteoprogenitor cells to avoid rejection, could provide a valuable approach to the repair of critical bone defects in the skull. However, in patients with syndromic craniosynostosis, autologous osteoprogenitors carry the mutations which cause premature ossification and consequent malformations requiring cranioplasty. The aim of this study was to develop strategies for growing effectively mutated osteoprogenitors with the view to developing autologous therapies for patients with craniosynostosis.

To this purpose, the behaviour of mouse osteoprogenitor cell lines carrying either a common human mutation (FGFR2-C278F) for syndromic craniosynostosis or the human wild type FGFR2 (WT-FGFR2) was investigated. In particular the focus was on i) how the mutation affected proliferation and osteogenisis; ii) the effect of exogenous fibroblast growth factor (FGF) 18 on cell growth and morphology; iii) analysis of osteoprogenitor attachment and growth on bioabsorbable scaffolds uncoated or coated with extracellular matrix molecules (fibronectin, laminin).

RT-PCR analysis of cells carrying the FGFR2-C278F mutation and wild type FGFR2 cells, revealed differential expression of FGF18 and molecules important in the terminal differentiation of mineralising osteoblasts: Osteocalcin was expressed at greater levels in cells carrying the mutation compared to wild type cells at 1.8 fold ($p < 0.01$) and 1.4 fold ($p = 0.02$) in pre-confluent and day 5 confluent cells respectively. Wild type cells expressed alkaline phosphatase from day 5 compared to mutated cells which did not express until day 30. Most striking was the greater expression of FGF18 in the mutated cells; 1.8 fold ($p < 0.01$) at pre confluence, 1.3 fold ($p = 0.01$) at day 5 and 1.5 fold ($p < 0.01$) at day 30.

In the early phase of cell cultures, cells carrying the mutation demonstrated higher mitotic activity than wild type cells as determined by p-H3 staining (18.6% +ve and 11.2% +ve respectively; $p < 0.01$, day 2 post plating). However, mutated cells demonstrated altered attachment behaviour, clustering forming early nodules and did not reach full confluence. Subsequent treatment of mutated cells with FGF18 [10^{-9}M] appeared to allow cells to reach confluence and “rescue” the phenotype. A concentration dependent increase

in cellular mitosis for all cell lines was observed. This was significantly greater at day 1 in mutated cells compared to wild type cells (51% and 29% respectively; $p < 0.01$ at FGF18 [10^{-9}M], and 60% and 43% respectively; $p < 0.01$ at FGF18[10^{-8}M]).

Attachment and growth of osteoblasts to a commercially available, clinically licensed scaffold could be enhanced by modification of the surface with the extracellular matrix (ECM) molecules, fibronectin and laminin. Both substrates significantly enhanced attachment at all time points up to 48 hours post plating (e.g. C278F cells at 1 hour post plating: 10,020 cells [-ve]; 37,600 [fibronectin]; 41,670 [laminin]; $p < 0.01$).

These findings indicated that the use of osteoprogenitor cells carrying mutations could present a feasible strategy in bone tissue engineering, which warrants further development towards the overall multidisciplinary approach in the management of these complex clinical challenges.

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Abbreviations

α-MEM	alpha modification of Eagle's minimal essential medium
ABC	avidin-biotin peroxidase complex
ALP	alkaline phosphatase
BCIP	5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine
BMP	bone morphogenetic protein
BrdU	5-Bromo-2′-deoxyuridine
BSA	bovine serum albumin
bp	base pair
CAM	cell adhesion molecule
cDNA	complimentary deoxyribonucleic acid
CMFDA	5-Chloromethylfluorescein diacetate
CS	Crouzon syndrome
Cx43	connexin 43
d	day
Da	dalton
DAB	diaminobenzadine tetrahydrochlorodihydrate
DEPC	diethylprocarbonate
DMSO	dimethyl sulphoxide
DNase	deoxyribonuclease
DPBS	Dulbecco's phosphate buffered saline
dNTP	deoxynucleoside triphosphate
ECM	extracellular matrix
EDTA	ethylene-diamine tetra-acetic acid
FBS	foetal bovine serum
FCS	foetal calf serum
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FN	fibronectin
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GOSH	Great Ormond Street Childrens Hospital
HSPG	heparan sulphate proteoglycan
Ig	immunoglobulin
IMS	industrial methylated spirit
JWS	Jackson-Weiss syndrome
kDa	kilodalton
L	DNA ladder
MS	Muenke syndrome
M-phase	mitosis phase
MMLV	Moloney murine leukaemia virus
MOPS	3-(N-morpholino) propanesulphonic acid
mRNA	messenger ribonucleic acid
n	number

NBT	Nitro-Blue Tetrazolium Chloride
OC	osteocalcin
PCR	polymerase chain reaction
pds	primer dimers
PF	Pfeiffer syndrome
PFA	paraformaldehyde
PGA	polyglactide (poly glycolic acid)
PLA	polylactide (polylactic acid)
PLGA	polyglactide/polylactide
PMM	polymethylmethacrylate
ER	endoplasmic reticulum
RE	restriction enzyme
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RT	reverse transcriptase
S	significant
SCS	Saethre-Chotzen syndrome
SD	standard deviation
SEM	standard error of mean
S-phase	synthesis phase
TAE	tris acetate EDTA electrophoresis buffer
TKD	tyrosine kinase domain
TMD	trans membrane domain
WT	wild type

Chapter 1: Introduction

This thesis presents a study of osteoprogenitor cells carrying a mutation in the human fibroblast growth factor receptor 2, coding for a syndromic form of craniosynostosis. It further explores the potential use of cells carrying a mutation for bone tissue engineering. The introduction will discuss the major themes of the study by giving an overview of:

- Craniofacial development and craniosynostosis
- The surgical difficulties in managing craniosynostosis
- Fibroblast growth factor receptors (FGFRs) and fibroblast growth factors (FGFs)
- Biomaterials in bone tissue engineering

These themes will then be consolidated to form the hypothesis for this study.

1.1: Cranial Development and Ossification

1.1.1: Origin of the Craniofacial Skeleton

The mammalian craniofacial skeleton accommodates the expanding brain and supports the structures and functions of existence such as feeding, breathing, seeing and social interaction. It is a composite of neurocranium (calvaria/vault) which forms principally in membranous bone and viscerocranium (skull base and facial skeleton) which forms by endochondral ossification via a cartilaginous precursor (**Fig. 1.1**). The membranous bones of the cranial vault are formed by ossification of mesenchymal condensations (Hall & Miyake 1992), derived from early populations of cephalic neural crest which have migrated from the closing neural folds (Noden 1975, 1983; Couly & LeDourian 1985; Tan & Morriss-Kay 1985) and mesoderm (Morriss-Kay 2001; Jiang et al., 2002; Jeong et al., 2004). The cranial bones lie between the dura mater which surrounds the brain and is derived from neural crest (Yu et al., 1997) and by aponeurosis and skin originating in the ectoderm. The sutures, which lie between the numerous bone plates of the skull vault and base, are formed by neural crest-mesoderm boundaries (Jiang et al., 2002).

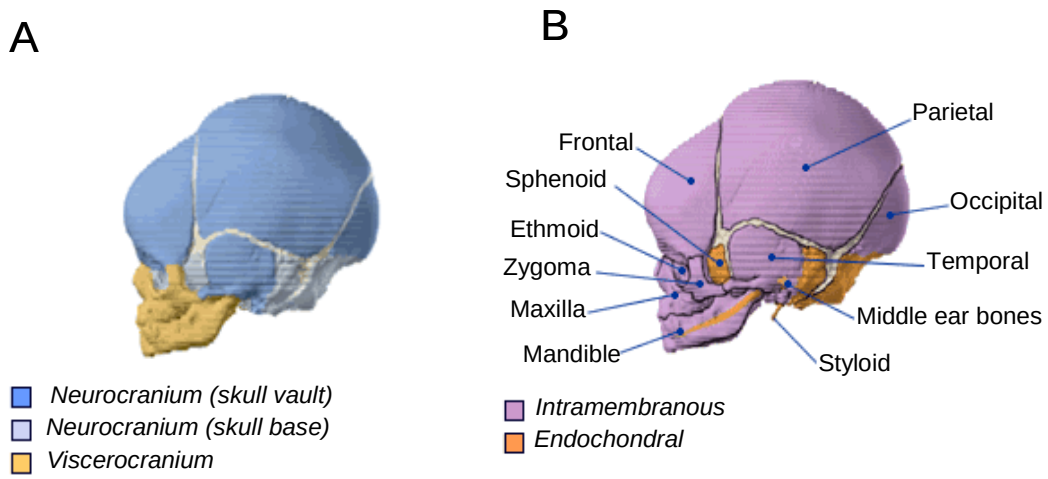


Figure 1.1: Development of the foetal cranium

A: Lateral view defining nomenclature boundaries.

B: Lateral view of foetal skull showing the principal bones and their mode of osteogenisis (adapted from: www.hopkinsmedicine.org/craniofacial)

1.1.2: Skull Morphology Sutures and Fontanelles

Sutures form the interface zones between two bones of the cranial base or calvaria, whilst fontanelles form where three or more bones meet. The principal sutures being the coronal sutures (paired) between the frontal and parietal bones, the metopic suture between the two hemi-frontal bones, the lambdoidal suture (paired) between the parietal and occipital bone and the sagittal suture between the two parietal bones. The anterior fontanelle exists at the confluence of the two parietal and frontal bones and the posterior fontanelle, at the confluence of the two parietal bones and the occipital bone (**Fig. 1.2**).

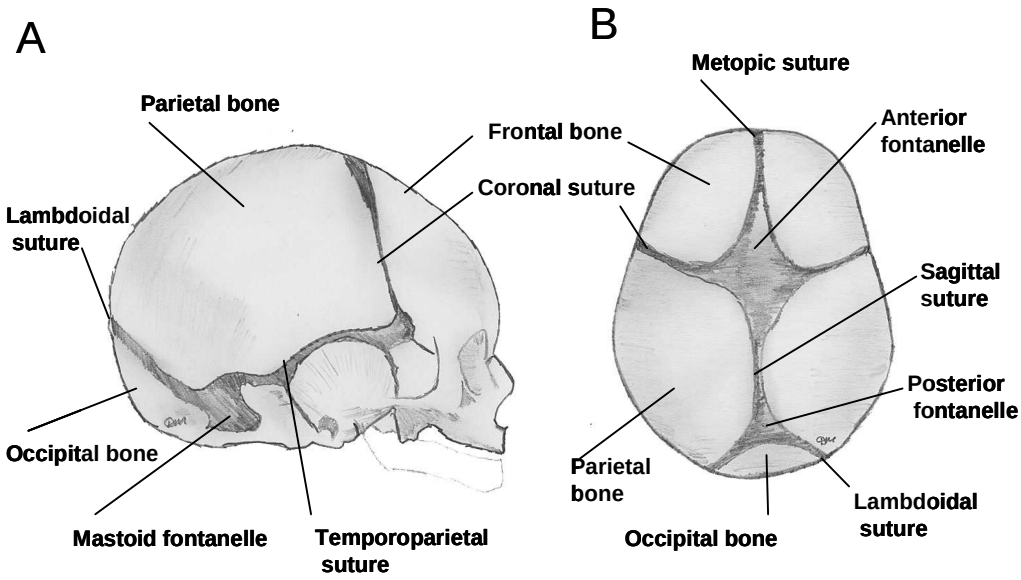


Figure1.2: Illustration of principal sutures and fontanelles of the foetal cranium

A: Lateral view of the skull representing approximately 25 weeks gestation and showing coronal, temporoparietal and lambdoidal sutures.

B: Antero-posterior view of the skull showing the occipital bone, paired parietal and frontal bones with intervening lambdoidal, sagittal and parietal sutures, expanded by the fontanelles. (Redrawn after Morriss-Kay & Wilkie 2005)

Sutures and fontanelles allow for a degree of compression of the skull during birth and subsequent growth and remodelling, driven principally by the expanding brain. Sutures are fundamental in allowing changes in form, size and orientation of the craniofacial skeleton in response to normal or pathological growth of adjacent structures. This occurs by a coordinated balance of bone deposition and resorption at the bone/mesenchyme/periosteal/dural interface. (**Fig. 1.3**)

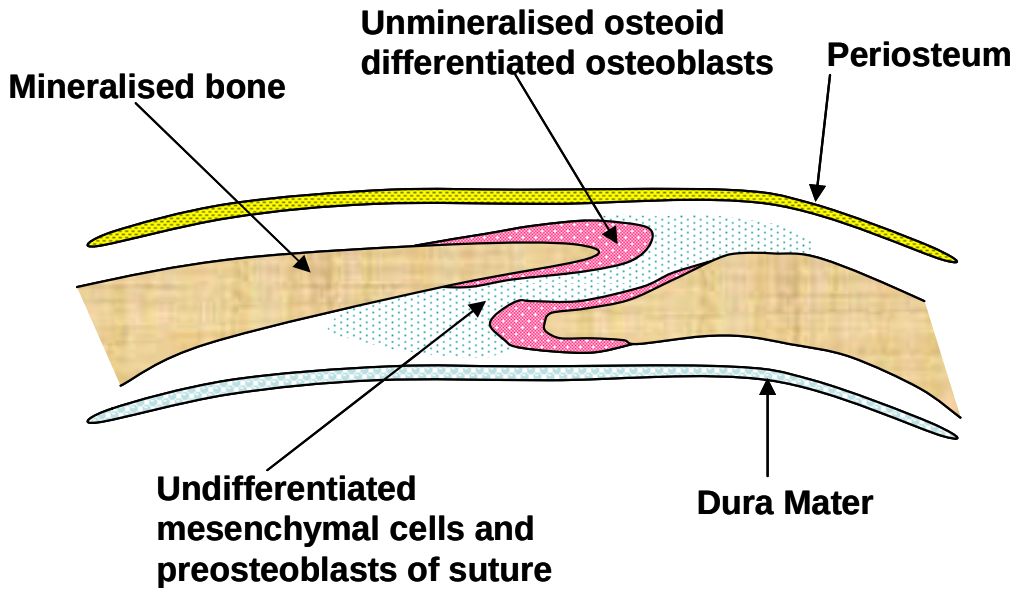


Figure 1.3: Schematic illustration of cranial suture

Diagrammatic section through a patent cranial suture. In the adult human these are normally obliterated in a temporal sequence (Table 1.1). In craniosynostosis, suture obliteration occurs prematurely and often in-utero. (Redrawn after Marie 2003)

Patency of the sutures is maintained during growth of the brain, which initially keeps the sutures widely separated (Cohen & McLean, 2000) and subsequently by signalling mechanisms within the premineralised mesenchymal zone. Cells within the suture area differentiate into osteogenic cells, and contribute towards the formation of new calvarial bone, whilst others persist in an undifferentiated, proliferative state (Cohen, 1993). Following birth, human sutures fuse in a site and time dependent manner which can be highly variable (Sahni, 2004), although generally earlier in males than females and commencing on the endocranial surface (Vu, 2001), consistent with findings in mice (Opperman et al., 1997; Roth et al., 1997). **Table 1.1** shows the normal sequence of suture fusion. Whilst most of the calvarial sutures remain patent through adulthood, the metopic suture closes within the first two years of life. This may be related to the finding that the metopic is the only suture which forms solely within neural crest domain, the others being formed at neural crest-mesodermal boundaries (Jieng et al., 2002).

Fontanelle	Timing of Closure
Posterior	0-4 months
Mastoid	6-12 months
Anterior	12-24 months
Suture	**Timing of Fusion**
Metopic	3-48 months
Sagittal	20-45 years
Lambdoidal	25-45 years
Coronal	25-50 years
Spheno-temporo-parietal	30-60 years

Table 1.1: Normal timing of human suture and fontanelle closure post-partum
(Created from data in: Perizonius, 1984; Vu et al., 2001; Sahni et al., 2004)

1.1.3: Dura mater

Sutures also require the proximity of the dura mater for sustained patency. Opperman et al., (1993, 1995), demonstrated that the dura initially plays an inductive role, then a permissive role in maintaining sutural patency through various signalling factors. Embryonic mouse dura has been shown to regulate sutural patency (Kim et al., 1998) and Levine et al., (1998), determined that the dura generates signals and that these signals could be blocked by a silastic sheet interposed between the dura and the overlying suture. Dura transplanted from under normally patent sutures to sites of sutural fusion in a naturally occurring craniosynostotic rabbit model, successfully kept these sites from re-obliterating following surgery (Mooney et al., 2001).

1.1.4: Mechanical forces

Mechanical forces may also play a role in maintaining sutural patency or premature fusion. It is a striking observation that skull size and form, matches brain size, in both children with normal brains and in those with hydrocephalus or microcephaly, a condition in which there has been an “appropriate” premature suture fusion. In this situation, a primary failure of the brain to grow is interpreted by poorly understood biofeedback mechanisms within the sutures, which then go on to fuse.

Fusion of the right coronal suture is twice as common as on the left, possibly reflecting the 3:1 right laterality bias of cephalic presentation births (Ververs et al., 1994). The application of tensile forces across rat sutures has been shown to cause an increase in the production of Insulin like growth factor receptors in osteoblast-like cells, resulting in cellular proliferation at the suture (Hirukawa et al., 2004).

Sutures thus allow the growth of the craniofacial skeleton, usually symmetrically and principally in response to the growing brain. However, if individual or multiple sutures fuse prematurely, the remaining patent sutures can respond by compensatory growth, often leading to asymmetry (Jane & Persing, 1986). Where sutures have fused prematurely, this is known as craniosynostosis and shall be discussed in further detail.

1.2: Craniosynostosis

1.2.1: Description of Craniosynostosis

Craniosynostosis is a defect which effects the normal temporo-spatial development of the sutures of the cranial vault and/or skull base. It follows failure of the signalling mechanisms that govern the processes of growth and differentiation at the suture margins. This results in the premature fusion of one or more of the cranial sutures. Fused sutures may be found in isolation (non-syndromic craniosynostosis), as part of a syndrome (syndromic or complex craniosynostosis), or as a secondary consequence of a primary disease process such as one of the metabolic disorders. The combined incidence of these disorders is approximately 1 in 2500 live births (Cohen & McLean, 2000).

Premature fusion of sutures prevents further bone apposition and hence growth of the bone perpendicular to the line of the suture. Continued and compensatory bone growth in those sutures which have remained patent, results in the development of an abnormal

skull shape (**Fig. 1.4**), the form of which depends upon the sequence and site of premature fusion. Traditionally, craniosynostosis is classified according to the sutures involved, the resulting dysmorphology of the head, the presence or absence of an identified craniofacial syndrome, and the degree of progression of the anomaly. Whilst the multi-suture and syndromic craniosynostosis do not lend themselves so readily to descriptive classification, specific head shapes are recognised most obviously in single suture craniosynostosis and a descriptive classification based on head shape exists (**Table 1.2).**

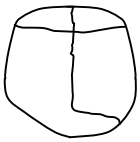
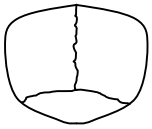
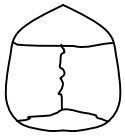
Affected Suture	Lambdoid	Sagittal	Uni-coronal	Bicoronal	Metopic
Clinical Classification	Posterior plagiocephaly	Scaphocephaly	Plagiocephaly	Brachycephaly	Trigonocephaly
Clinical Features	Flattened occiput Asymmetric ear position, Tilted head position	Antero-posterior elongation of the head Narrowing across temples	Asymmetric flattening of forehead Raised supraorbital rim on affected side	Symmetric loss of forehead contour Commonly associated with a syndromic form (Seathre-Chotzen)	Pointed forehead
Diagramatic Form					

Table 1.2: Clinical classification of single suture craniosynostosis

Adapted from Posnick (1999)

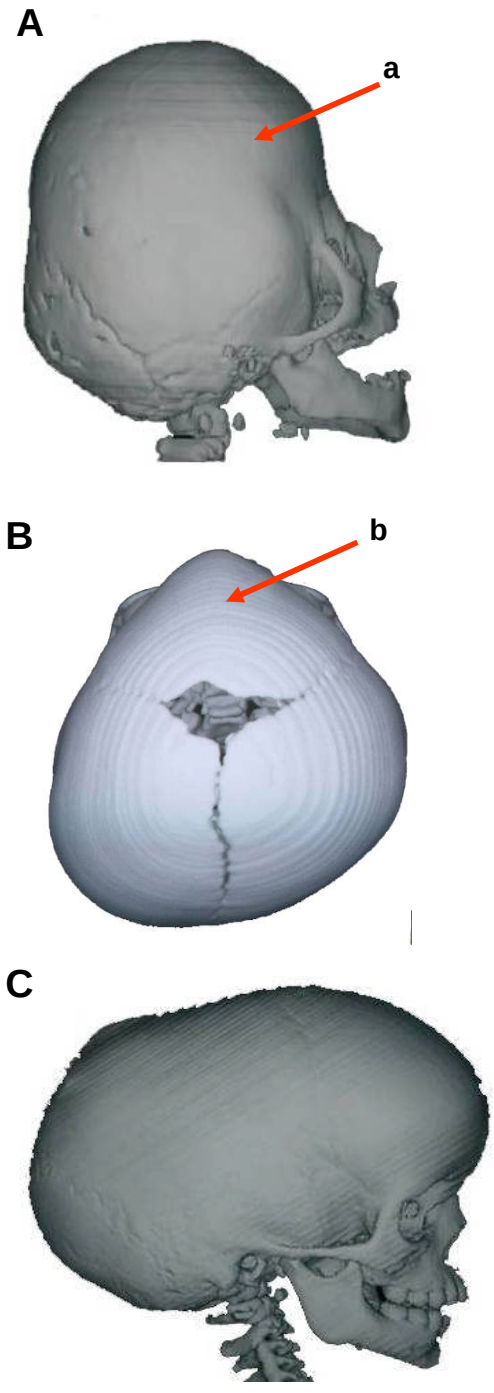


Figure 1.4: 3-Dimensional computer tomograms(CT) of craniosynostosis

A: Lateral view of a six month old child with bicoronal synstosis associated with Muenke syndrome. Arrow "**a**" denotes the location of the fused (absent) coronal suture.

B: Axial view of a 1 year old child with metopic synostosis. Arrow "**b**" denotes the location of the fused metopic suture.

C: Lateral view of a two year old child with sagittal synostosis. The fused suture is not visible in this view which demonstrates the typical antero-posterior elongation of the skull

(Great Ormond Street Hospital)

1.2.2: Aetiology of Craniosynostosis

The causes of craniosynostosis are numerous and involve a wide spectrum of conditions. The majority are not syndromic, but sporadic, isolated, single suture synostoses, where no molecular abnormality can be identified, and where it is likely that their aetiology is not of genetic origin (Cohen & McLean, 2000). Craniosynostosis can occur secondary to pathogenesis such as metabolic diseases e.g. hyperthyroidism, haematological disease, such as thalassaemia, glycogen storage disorders such as Hurler syndrome, and foetal teratogenesis induced by drugs such as retinoic acid and phenytoin (Gorlin et al., 2001; Thompson et al., 1994). Over 100 forms of causally related craniosynostosis have been described (Cohen & McLean, 2000). However, in up to 15% of cases of craniosynostosis there is evidence of Mendelian inheritance (Reardon & Winter, 1995). For historical reasons, these have been named eponymously by those first given credit for describing the clinical features of the syndrome. The most common craniosynostoses or syndromes with a defined molecular basis are, Seathre-Chotzen syndrome (1 in 20,000 live births) Crouzon syndrome (1 in 25,000), Jackson-Weiss syndrome (1 in 26,000), Apert syndrome (1 in 100,000), Pfeiffer syndrome (1 in 200,000) (Gorlin et al., 2001). In addition, the recently described Muenke syndrome which presents as a highly variable, atypical syndrome which is not easily categorised on clinical grounds (Muenke et al., 1997), accounts for between 17% and 31% of apparently non-syndromic coronal or bicoronal synostosis (Moloney et al., 1997; Lajeunie et al., 1999). The molecular mechanisms underlying these genetically based syndromes will be explored more fully later in this chapter.

1.2.3: Clinical Manifestations of syndromic craniosynosis

A number of presenting features are shared between the syndromes, these are summarised for the commonest syndromes in **Table 1.3**. These features are used to distinguish one syndromic case from another in clinical terms.

An abnormal skull shape [turricephally (tall flattened forehead), acrocephally (cone shaped) or “klebblatschadel” (clover-Leaf), widely spaced orbits (hypertelorism)], proptosis and an underdeveloped midface with apparent prognathism are characteristic features of these disorders. Hand and foot anomalies are observed in some, but not all craniosynostosis syndromes. The severest digital anomalies are found in Aperts syndrome and involve variable but significant degrees of syndactely (fusion of the digits) which usually require surgery to improve function (Upton, 1991), Children with Saethre-Chotzen

syndrome have variable and often subtle degrees of sydactely of the 2nd 3rd or 4th web spaces, whilst children with Pfeiffer syndrome have broad thumbs and broad big toes which are deviated towards the midline. They may also demonstrate mild levels of webbing between the digits of the hands and feet. Jackson-Weiss syndrome demonstrates foot abnormalities very similar to Pfeiffer syndrome, but interestingly the digits of the hand are unaffected. (Cohen & McLean, 2000)

Phenotype	AS	PS	CS	SCS	JWS
Craniosynostosis	+	+	+	+	+
Midface hyposplasia	+	+	+	-	+
Hand anomalies	+	+	-	+	-
Foot anomalies	+	+	-	+	+

Table 1.3: Phenotypes in craniosynostosis syndromes.
AS: Apert syndrome, **PS**: Pfeiffer syndrome **CS**: Crouzon syndrome, **SCS**: Saethre-Chotzen syndrome, **JW**: Jackson-Weiss syndrome,(Muenke syndrome has been omitted as the clinical phenotype is less severe). Adapted from Muenke and Schell, (1995)

The clinical phenotype is not limited to premature fusion of the cranial sutures and defective limb development. Individuals may present with additional cutaneous (Orlow, 1992; Hall 1992), dental (Berkowitz 1971; Sidhu 1989), ophthalmic (Matthews, 1979), aural (Bergstrom et al., 1972), cardiac (Hunter & Rudd, 1977), respiratory (Gonzalez et al., 1997) and CNS abnormalities (Davidson & Young, 1981; Thompson et al., 1997). As a result of this spectrum of potential clinical manifestations, children with craniosynostosis suffer from a variety of often serious functional complications. These include airway obstruction, due to midface hypoplasia (Lauritzen et al., 1986; Gonzalez et al., 1996), hydrocephalus (Murtagh & Baird, 1961; Noetzal et al., 1985), intracranial venous hypertension (Sainte-Rose, 1984; Thompson et al., 1995), raised intracranial pressure (Lundar and Hornes, 1991; Gault et al., 1992; Hayward et al., 1992; Gosain et al., 1995, Murovic et al.,1993; Thompson et al., 1995), palatal clefting (Cohen et al., 1971) and occlusal anomalies (Kreiborg & Cohen, 1992), visual problems such as proptosis, keratopathy and papilloedema (Miller 1984; Newman 1991), speech and language delay (Shipster et al., 2003) and developmental delay (Patton et al., 1988; Renier & Marchac 1988). In addition, psychosocial problems are increasingly recognised (Pertzchuk & Whitaker, 1987; Endriga & Kapp-Simon, 1999).

Raised intracranial pressure (ICP) represents one of the most serious consequences of syndromic craniosynostosis. Raised ICP can cause impaired cerebral perfusion and retinal damage, leading to blindness (Stavrou et al., 1997), impairment of intellectual development (Renier et al., 1982; Bellew et al., 2005), seizures, and can occasionally lead to premature sudden death in this group of individuals (Gosain et al., 1995). The incidence of raised ICP in individuals with syndromic craniosynostosis is at least twice as high as in non-syndromic cases. In addition to raised intracranial pressure, high-pressure hydrocephalus is prevalent amongst individuals with syndromic craniosynostosis. Hayward and Gonzales, (2005), have concluded that cerebral perfusion, raised intracranial pressure and respiratory obstruction interact in a viscious cycle to exacerbate neurodevelopmental damage. These studies confirm that raised intracranial pressure is a significant feature of syndromic craniosynostosis. The causes of raised intracranial pressure in these individuals remain highly contentious. However, vault constriction through premature sutural fusion is thought to play a fundamental role (Renier et al., 1982).

1.2.4: Surgical Options for Craniosynostosis

Therapeutic intervention for craniosynostosis involves surgery as the mainstay and surgical procedures are performed for a number of indications. The timing, extent and type of procedure is determined on the basis of the severity and location of the cranial deformity. Early surgery would undoubtedly be desirable in order to correct deformity and prevent functional problems as previously described. However, continued abnormal growth and ossification would in a high proportion of cases lead to relapse and further, possibly earlier episodes of surgery (Hayward et al., 2004). The planning of surgery tends to fall into two categories: Those procedures carried out electively for psychosocial wellbeing and elective functional problems such as severe malocclusion and those which can be considered as being performed on a “crisis intervention” basis to alleviate or prevent progressing functional deficits. Concerns over increased intracranial pressure strongly influence the timing and staging of surgical intervention. This latter group is for the vast majority associated with syndromic cases with multiple suture involvement or pansynostosis, although raised intracranial pressure and its sequelae, can occur in single suture craniosynostosis (Renier et al., 1982; Gault et al., 1992).

1.2.4.1: Vault Expansion Procedures

The purpose of early calvarial surgery in syndromic craniosynostosis is two-fold; firstly lowering of raised intracranial pressure and venous hypertension by immediate expansion of the intracranial volume and secondly, removal or release of fused sutures to allow further unconstrained growth of the brain. Furthermore, combined fronto-facial advancement procedures (Ortiz-Monesatrio, 1978) may be required at an early stage to produce an increase in intracranial volume, simultaneous with an advancement of the facial skeleton for protection of exposed cornea and improvement in respiratory function, by opening of the nasopharyngeal airway (Polley et al., 1995; Britto et al., 1999).

Surgical procedures for individuals with complex craniosynostosis include cranial vault expansion or fronto-facial advances. Vault expansion can be accomplished either as a staged procedure or in a single stage. There are proponents of each technique and no individual approach has been proven superior in a validated way. However, most authors favour a two stage procedure with either primary anterior or posterior vault decompression, followed by counterpole vault expansion (Marchac et al., 1995; McCarthey et al., 1995; Hayward et al., 2004). Others, less commonly, advocate a complete single stage calvarial vault expansion (Persing et al., 1981; Zuccaro et al., 1996).

Recently the technique of distraction osteogenisis has been added to the armamentarium of the craniofacial surgeon (McCarthey, 1992; Cohen et al., 1999; McCarthey et al., 2001). This has allowed larger and more stable advances of the craniofacial skeleton to be achieved with concomitantly fewer serious side effects such as life threatening infection (Cohen et al., 1999; Shin et al., 2003). However, while this technique has significant advantages, this has also led to the simultaneous creation of large bony defects overlying the brain (Bradley et al., 2006 and personal observations), which are filled with a thin fibrous scar joining the dural surface to pericranium or galeal aponeurosis (Dahlin et al., 1991; Ripamonti, 1992) (**Fig 1.5**). However, there is also a period of soft tissue interposition prior to successful regeneration of long bones through distraction osteogenisis. Why bone does not regenerate as completely in distracted cranial bones as it does in long bone is not fully understood. There is evidence that distraction osteogenisis in long bones proceeds when cells are stressed (Kanno et al., 2005; Cho et al., 2007) which in evolutionary terms, links to the fact that long bones are undergoing significant stresses during normal function, whereas the cranium is a protective vault compiled of normally unstressed bony plates.

Of further importance may be the fact that cranial bone is derived from neuroectodermal origin and forms in membrane without going through a cartilaginous

precursor. The recent work by Leucht et al., (2008), who have highlighted the potential clinical importance of fundamental differences in expression of genes such as the *Hox* patterning genes between mesenchyme derived and neuroectoderm derived osteoprogenitors, may provide vital clues to direct the future manipulations aimed at resolving the problem of cranial bone regeneration.

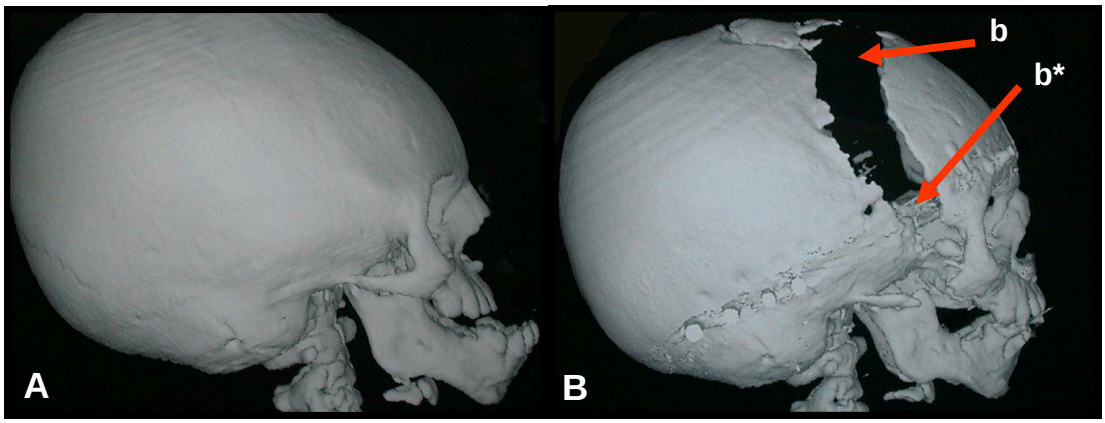


Figure 1.5: Post fronto-facial advance for child with Crouzon syndrome

A: Lateral skull view of 3-D CT scan showing a two year old boy with Crouzon syndrome one month before surgery. This demonstrates the relative mandibular prognathism which is a result of poor forward and downward growth of the orbito-maxillary complex due to suture fusions of the neuro and viscerocranium.

B: Similar view 2 months post surgery. Monobloc osteotomies were performed followed by a one week period to allow callus formation and soft tissue healing before daily distraction of the fronto-facial skeleton by 1mm for 25 days. A further four week period of consolidation is necessary before frame removal. Arrow **b*** points to bony regeneration which has occurred in the vicinity of the skull base and mid-facial bones. Arrow **b** demonstrates the absence of bone regeneration of the cranial vault. Minimal further regeneration of bone would be anticipated in the vault regions in a child of this age or older. (*Great Ormond Street Hospital for Children*)

1.2.5: The surgical problem.

The precedent and to a lesser extent the subsequent review, has been carried out with the focus on reconstruction of the craniofacial skeleton, as it relates to the conditions and surgical challenges encountered by the craniofacial surgeon. However, scenarios involving replacement of bone are also a frequent and sometimes everyday occurrence for orthopaedic surgeons, neurosurgeons, otolaryngologists and oral surgeons. The review therefore has relevance to all of the surgical specialties involved in bony reconstruction. This is manifest in the broad source of the review material, drawn from all of the above specialties and the basic scientific literature.

By the very nature and geometry of vault expansion, what all surgical approaches have in common is that defects will be created between the cut edges of the expanded bone. Moreover, spaces may be created between the dura and the dural surface of the calvarial bone (Wolfe et al., 1993). These spaces may eventually be obliterated by the expanding brain. However, this remains a potential cause of poor bone repair, as the dural-bone interface has been shown in animal studies to be the source of greatest bone regeneration (Hobar et al., 1993; Gosain et al., 2003). These studies also demonstrated that regeneration is significantly poorer in mature animals. This is consistent with findings in humans, where the capacity of children over the age of 2 to regenerate calvarium is very limited (Sirola, 1960; Bradley et al., 2006). This may be linked to the findings that immature dural cells are capable of producing greater amounts of growth factors compared with mature cells. (Greenwald et al., 2000).

The knowledge that calvarial defects do not heal in all but the youngest children, has necessitated the repair of defects with a number of alternatives. This is required for a) protecting individuals from the risk of cerebral injury, b) stabilisation and prevention of relapse in the “craniotomised” bony fragments and c) optomising aesthetic outcome by restoration of contour defects.

1.2.6: Current techniques for calvarial defect reconstruction

Historically, autologous bone grafts, allografts, and a variety of alloplastic biomaterials have been used for the repair of cranial defects (Koenig et al., 1995; Gosain et al., 1999). However, problems related to graft availability, donor site morbidity, unpredictable graft resorption (**Fig.1.6**), long term growth, long-term biocompatibility, immunogenicity, and biomechanical integrity, have all limited the success and applicability of these methods (Gosain & Persing, 1999; Pou, 2003), Moreover, because alloplastic materials remain as permanent foreign bodies, their use in pediatric patients is limited by concerns about their influence on the growing craniofacial skeleton, including the possibilitiy of non degradable materials being translocated to the endocranial surface (Fearon et al., 1995).

1.2.6.1: Autologous Bone Graft

Several techniques and material options for the reconstruction of calvarial defects exist. The choice made should take into account many factors including surgical site, risk of infection, previous surgeries, indication for surgery and patient age, to account for the need for future growth (Moreira-Gonzales et al., 2003). The most commonly used technique available for reconstruction of calvarial defects is the autologous free bone graft. Autologous grafting for craniofacial defects was first reported by Muller in 1890 to restore a skull defect by harvesting bone from the cranium (van Wyck, 1989). Since then and despite drawbacks, autologous grafts have remained popular and are considered by most specialists to be the gold standard in the absence of the “ideal” bone substitute (Citardi & Friedman, 1994; Kirschner et al., 2002). The most commonly utilised donor areas include cranium, rib, and iliac crest grafts (Guyuron et al., 1988; Prolo & Oklund, 1991). They have the advantage of bringing potentially osteogenic tissue to the defect and in-growth of adjacent fibro-vascular tissue (Gladstone et al., 1995; Jackson et al., 1987). However, they are not vascularised tissues in themselves and the degree to which they are incorporated is variable. Cranial bone grafts have the advantage of being situated at the site of primary surgery, thereby avoiding the need for a further donor site. Often large amounts of bone can be harvested by splitting of the calvarial graft into an inner and outer cortex. However, this technique is dependent on there being a well developed, age related diploe between cortices to allow feasible splitting (Koenig et al., 1995). Unfortunately, this is seldom the case in individuals with craniosynostosis who need surgery at an early age.

Bone grafts from all donor sites are prone to unpredictable but significant levels of resorption (Moreira-Gonzales et al., 2003), although cranial bone grafts are accepted as being the most reliable (Whitaker et al., 1979; Tessier 1982; Jackson et al., 1986; D'Adonna and Nowzari, 2000). The reliability of cranially derived graft may be linked to the recent findings of Leucht et al., (2008), who have shown in mice that mandibular defects regenerate more effectively when grafted with bone from a neuroectodermal origin. Suggesting that skeletal stem cells have 'positional memory', which influences how the cells behave when grafted into ectopic locations.

In addition to resorption or loss due to infection, complications associated with graft harvest can involve persistent cerebro-spinal fluid leak, epidural or subdural haematoma, contour deformities of the cranial vault, and permanent neurologic impairment (Kline & Wolfe 1995; Zins et al., 1995). Iliac crest grafts are readily available, but they have been associated with persistent postoperative pain, herniation of abdominal contents, haematomas, and neuropathy of the lateral femoral cutaneous nerve (Fowler et al., 1995; Russell & Block, 2000). Split rib grafts provide bone which is malleable to a degree and which complies well with skull form and in keeping with other autologous donor sites, it seldom fails due to infection. However, harvest involves a secondary donor site which can cause severe post operative pain or pneumothorax (Munro & Guyuron, 1981). In common with iliac grafts, rib grafts have been reported as undergoing a greater degree of resorption than cranial grafts (Zins & Whitaker, 1983; Hardesty & Marsh, 1990; Donovan et al., 1993), although recent studies in rabbits have found no difference in resorption volume between endochondral (iliac crest) and membranous (mandible) sources, when cortical bone was utilised, concluding that bone architecture dictates resorption and not embryological origin (Ozaki & Buckman, 1998).

1.2.6.2: Bone-Fibrin Composite

Where small defects are to be filled or where extensive surgery has produced significant quantities of bone dust or small bone fragments which can be morselised, defects can be filled by a bone pate, created by mixing the residual bone with commercially available fibrin glue (Anderl et al., 1987; Matsumoto et al., 1998). While this utilises bone which might otherwise be wasted, there are no studies confirming the long term outcome of a morselised bone composite, although a high resolution CT scan study, has confirmed the complete resorption of bone dust replaced during cranioplasty in all patients (O'broin et al., 1997)

1.2.6.3: Bone Allografts

Allogenic bone is non-vital bone taken from one individual and transferred to another individual of the same species. Bone allografts exist in three main forms; a) fresh frozen, b) freeze dried and c) demineralised bone matrix (DBM). Due to concerns regarding transmission of viral and PRION infection, only DBM continues to have a significant role to play in the clinical setting. Demineralised bone matrix is produced by demineralisation of freeze dried bone, a process which not only removes any inherent structural strength but also renders the material incapable of transmitting infection (Mellonig et al., 1992). Urist (1965), demonstrated in the 1960s that DBM induced the formation of bone in non-osseous tissue such as muscle. This ability to induce osteogenisis in cells, not of the osteogenic lineage is termed osteoinduction. It has subsequently been shown that this osteoinductive ability is due to the availability of bone morphogenic proteins (BMPs) following the demineralisation process (Urist et al., 1984; Wozney et al., 1988). DBM has been shown to provide complete bone healing of critical-sized defects in rabbits (Lindholm et al., 1993) and dogs (Oklund et al., 1986). Despite the fact that commercial formulations of DBM have been available since 1991, recent studies have reported great variability and inconsistency with the levels of BMPs present and ability of the material to heal bony defects (Maddox et al., 2000; Russell et al., 2000). DBM is used widely for a number of dental (Callan 2000, Babbush 2003) otolaryngological (Leatherman & Dornhoffer, 2004) and orthopaedic indications, particularly spinal scenarios (Ludwig & Boden, 1999; Vaccaro et al., 2002; Price et al., 2003). However, the take-up in craniofacial practice appears limited, although Chen & Wang (2002), have reported favourable results in 10 consecutive patients with an average 33 months follow-up.

1.2.6.4: Polymethylmethacrylate

Polymethylmethacrylate (PMM) or PMM hybrid cements are widely used and considered to be one of the most consistently accepted options for reconstruction in the fully grown skull (Blum et al., 1997; Lara et al., 1998). They are however, not resorbed, incorporated or replaced by bone and do not adapt with the growing skull. They are difficult to model during polymerisation which involves a highly exothermic reaction (peak temps of 81^{o}C), potentially causing thermal necrosis to the underlying tissues (Stelnicki & Ousterhout, 1996). Giant cell foreign body reactions have been described (Constantino et al., 1992) and may cause loosening and fragmentation, necessitating removal. Removal due to infection is not common unless associated with the sinuses or multiple procedures (Manson et al., 1986).

1.2.6.5: Titanium

While autologous bone remains the preferred method of reconstruction for cranial defects, the reconstruction of large defects becomes more difficult owing to the availability of appropriate bone stock. In these circumstances defects can be reconstructed with prefabricated titanium plates (**Fig. 1.6**) or mesh (Simpson, 1965). Until the early 1990s this technique had the disadvantage of requiring a two stage procedure in order to construct an accurately fitting prosthesis, the first stage allowing an accurate impression of the defect to be obtained. This problem has since been overcome by the ability to model the titanium prosthesis from 3-Dimensionally reformatted CT scans and stereolithographic models (Joffe et al., 1992, 1999). Titanium is inert and does not produce a significant foreign body reaction and reports of removal due to displacement or infection are few (Joffe et al., 1999; Kamyszek et al., 2001). They are however not suitable for use in the growing skull.

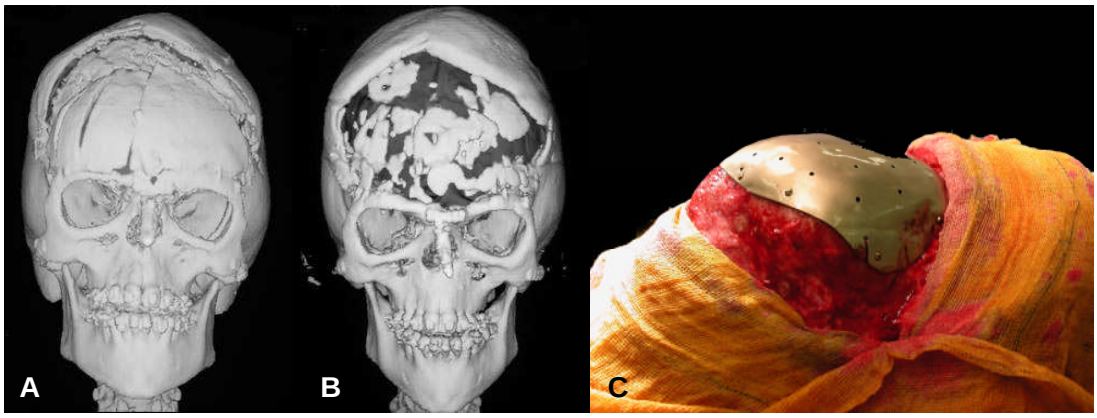


Figure 1.6: Bone resorption and prefabricated titanium plate reconstruction

A: A 17 year old with Apert syndrome underwent recontouring of the frontal region with split calvarial grafts.

B: Six months following surgery and associated with a chronic staphylococcal infection of the bone, the majority of grafted bone has resorbed, creating a further significant contour defect and resulting in only soft tissue protection for the brain. **C:** Lateral view (patient supine) of exposed skull. The brain is protected and contour defect reconstructed using a prefabricated titanium plate due to risk of further resorption and further graft availability. (*Great Ormond Street Hospital for Children*)

1.2.6.6: Calcium salt compounds

In the search for bioinert, osteoactive, easily applied and adaptable alloplastic materials, ceramic forming, calcium salt compounds, used separately or in combinations, have gained popularity (Burstein et al., 1999; Gosain et al., 2002; Durham et al., 2003; Eppley, 2003). Commercially produced hydroxyapatite [$Ca_{10}(PO_4)_6OH_2$] is chemically similar to the mineral component which accounts for 70% of the mineralised tissue in mammals. Furthermore, it is naturally occurring, tissue compatible, radiolucent and readily available.

Biologically, hydroxyapatite and other ceramic compounds are meant to substitute for the mineral phase of bone and are purely osteoconductive, although they can be combined with autologous cancellous bone to provide osteogenic potential (Ohgushi et al., 1989; LeGeros 2002). Their ability to support osteoconduction seems to be dependent upon porosity, which appears to be important in allowing diffusion of nutrients, cellular attachment and tissue ingrowth (LeGeros, 2002). Typically, these bioceramics degrade by a combination of mechanisms; a) simple physicochemical dissolution and b) osteoclastic, resorption (LeGeros, 1993; Pietrzak & Ronk, 2000). Tay et al., (1999), recommend that the use of these materials should be limited to the filling of bony defects in which the surrounding host environment is conducive to bony healing.

Comparison of studies reporting the use of these compounds is difficult, as they relate to a large number of different formulations, studied within different species, observing differently sized defects. Of the many studies which exist, reports have been mixed. Animal experiments have reported generally favourable findings (Kirschner et al., 2002). Moreira-Gonzales et al., (2003), reported good results with the use of hydroxyapatite granules for augmentation in the maxillary-mandibular region. The same authors reported that hydroxyapatite paste for reconstruction of human calvarial defects produced an immunoguided delayed inflammatory reaction that leads to thinning of the skin and exposure of the material, making secondary repair difficult. High levels of loosening, fracture, infection and extrusion have also been reported. (Matic & Manson, 2004),

The most challenging surgical cases in terms of bony reconstruction are those with syndromic craniosynostosis. Since the aetiology which underlies the majority of these conditions is abnormal signalling via the fibroblast growth factor receptor (FGFR) group of receptors and their ligands the fibroblast growth factors (FGFs), it follows that any attempts at cell based tissue engineering strategies, are likely to be influenced by these mutations and interactions also.

1.3: Fibroblast growth factors and receptors

1.3.1: Fibroblast growth factors

The fibroblast growth factors (FGFs) are a family of ligands which bind and activate fibroblast growth factor receptors. The FGF family consists of structurally related low molecular weight polypeptides of which 22 have been identified in mammals (Ornitz & Itoh, 2001). Features of FGFs include a homologous central core of 140 amino acid residues which form a compact β-barrel, similar in structure to interleukins 1α and 1β (Smallwood et al., 1996), with a strong affinity for heparin and heparan sulphate proteoglycan molecules (Schlessinger et al., 1995). Most FGFs are readily secreted from cells. However, the most ubiquitous factors, FGF1 and FGF2 lack a secretory amino acid signal sequence and are not secreted, but enter the extracellular matrix and cellular surfaces by a mechanism independent of the endoplasmic reticulum-Golgi pathway (Mignatti et al., 1992).

Fibroblast growth factors are capable of inducing a wide variety of effects, such as angiogenesis, chemotaxis, cell survival, proliferation and differentiation in neuroectodermal, epithelial and mesodermal cell types (Basilico & Moscatelli, 1992; Szebenyi & Fallon, 1999). Studies together with analyses of the role played by FGFs in specific developmental systems and the analyses of the expression patterns of FGFs in different tissues and cells, demonstrate that FGFs play critical roles during most stages of mouse development and organogenesis (Ornitz & Itoh, 2001). Their pattern of expression is highly variable ranging from the widely expressed FGF2 (Bikfalvi et al., 1997) to the highly restricted expression of FGF4 (Niswander & Martin, 1992). However, we know that the expression of FGFs and their receprtors, is temporally and spatially regulated which is of importance during craniofacial ossification (Delezoide et al., 1998).

1.3.2: Fibroblast growth factor receptors

Fibroblast growth factor receptors (FGFRs) are members of the Immunoglobulin (Ig) superfamily of receptors that have been shown to play a key role in developmental processes including proliferation, migration and differentiation of tissues as well as being expressed at high levels during embryogenesis (Peters et al., 1994).

First discovered in the mid 1980s (Olwin et al., 1986), four FGF receptors (FGFR1-4) have been characterised. The general structure of FGFRs is illustrated in **Fig. 1.7**. The extracellular (ligand-binding) domain of FGFR is composed of three immunoglobulin (Ig) like domains, designated IgI, IgII and IgIII, stabilised by disulphide bridges. These intramolecular disulphide bridges result from an interaction between two highly conserved

cysteine residues, which stabilise the loop conformation. Situated between IgI and IgII is a region of 4-8 acidic amino acid residues, called the 'acid box' and a positively charged region in IgII that serves as a binding site for heparin. Also in this region is the 'cell adhesion molecule' (CAM) homology domain which is a stretch of 20 amino acids that shares sequence homology with NCAM and N-Cadherin. FGFRs have a single transmembrane domain (TMD) (Jaye et al., 1992) and, with the exception of the as yet uncharacterised FGFR5 (Kim et al., 2001), a 'split' intracellular tyrosine kinase domain separated into two parts by a 14 amino acid insert.

1.3.3: FGFR isoforms

An important feature of the FGFR family of receptors is that a variety of FGFR isoforms are generated by alternative splicing of FGFR transcripts. The different FGFR isoforms include FGFRs with an extracellular domain composed of either two or three Ig-like domains, soluble secreted FGFR forms, as well as alternative splicing in the third Ig-like domain (IgIII) that profoundly alters ligand-binding specificity (Miki et al., 1992). The alternative splicing in IgIII exists in FGFR1, 2 and 3, but not in FGFR4. It has been shown that exon 7 of FGFR2 gene encodes for the N-terminal half of IgIII (designated 'a'), while exons 8 and 9 alternatively encode for the C-terminal half of IgIII. and are thus designated as 'b' and 'c' forms of FGFR, respectively (**Fig. 1.7**). Splice variants alter receptor function, and in several cases, lead to different ligand-binding specificities. Structural and functional diversity amongst FGFR isoforms, combined with numerous receptor ligands of which fibroblast growth factors (FGFs) are the most important, results in the potential for extremely complex and diverse FGFR-ligand interactions (Ornitz & Itoh, 2001). It has been shown that the FGFR2b isoform is exclusively expressed in epithelial cells and that the FGFR2c is expressed exclusively in mesenchymal cells (Orr-Urtreger et al., 1993). The lineage-specific expression of the IIIb and IIIc isoforms of FGFRs enables interaction between the epithelial and mesenchymal layers during development in response to different ligands.

1.3.4: FGFR / ligand specificity

The extracellular regions of FGFRs are involved in ligand binding. Loops II and III and the interlinker region between them are required for conferring specificity upon the interaction between the receptor and FGF (Plotnikov et al., 1999). Ornitz et al., in 1996, showed that mitogenic responses to FGF signalling were highly specific, and dependent

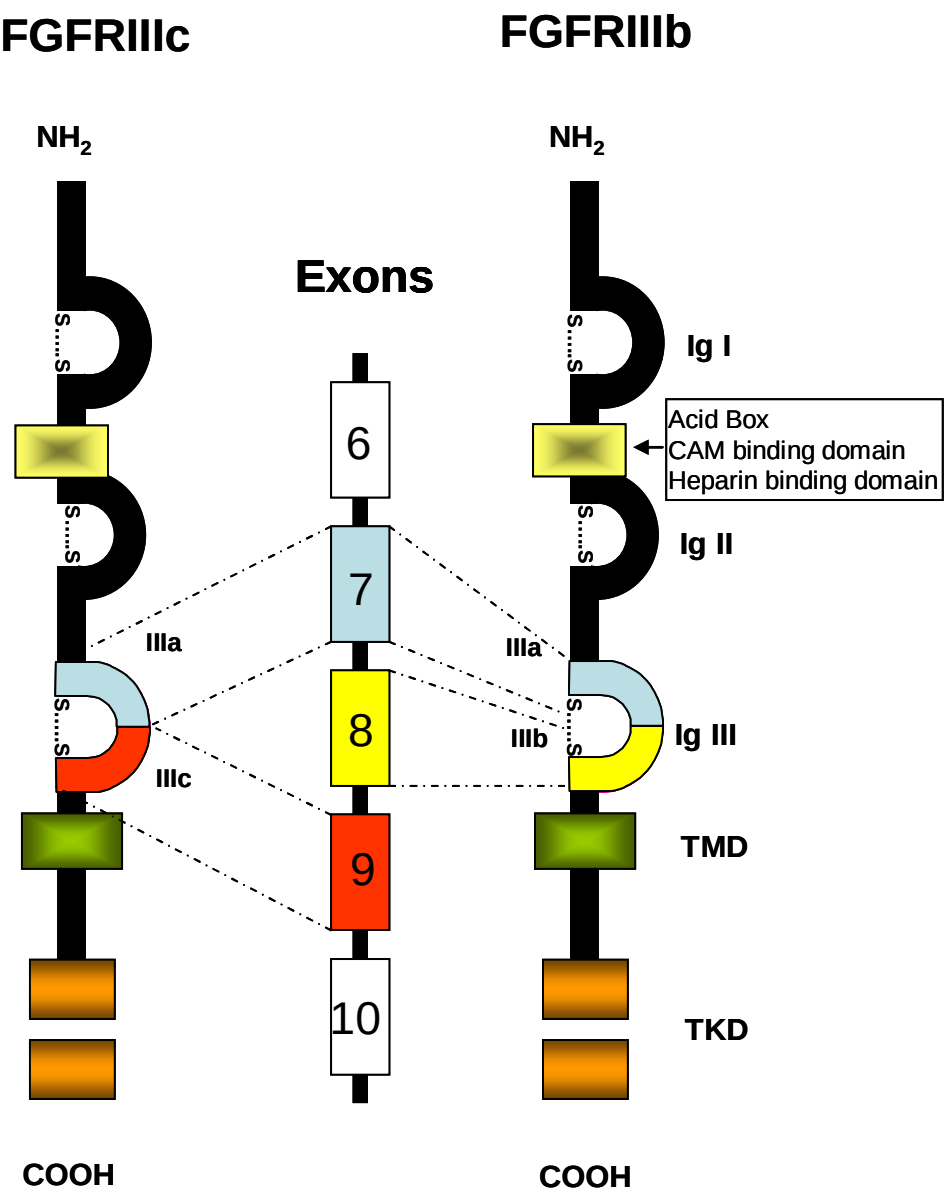


Figure 1.7: General structure and isoforms of FGFRs

FGFR isoforms generated by alternative splicing of FGFR transcripts. The two forms of FGFR are generated by alternative splicing of exons 8 and 9. The C-terminal half of IgIII is encoded by exon 8 to generate the FGFR-IIIb isoform while the C-terminal half of IgIII when encoded by exon 9 generates the FGFR-IIIc isoform. **TKD**= tyrosine kinase domain, **TMD**= transmenbrane domain (Adapted from Morriss-Kay & Wilkie 2005)

upon the combination of ligand and FGFR isoform. **Table 1.4** shows the specificities of FGFRs and FGFs based principally on this study.

Upon ligand binding, receptor dimerisation brings the cytoplasmic domains of the receptors in close vicinity of each other providing the opportunity for receptor trans-autophosphorylation, subsequent tyrosine kinase activation and initiation of downstream signalling pathways (Yarden and Ullrich, 1988).

The binding of FGFs to their receptors and subsequent dimerisation is dependent upon the low affinity, co-binding of heparan sulphate containing proteoglycans (Ornitz et al., 1992; Spivak-Kroizman et al., 1994).

FGFR Isoform	Ligand Specificity
1b	**FGF1,2,3,10**
1c	**FGF1,2,4,5,6**
2b	**FGF1,3,7,10,22**
2c	**FGF1,2,4,6,9,17,18**
3b	**FGF1 and 9**
3c	**FGF1,2,4,8,9,17,18,23**
4	**FGF1,2,4,6,8,9,16,17,18,19**

Table 1.4: Ligand specificities of FGFR isoforms

FGF ligand binding specificity determined by thymidine incorporation. Adapted from Ornitz et al., (1996); Xu et al., (2000) ; Yamashita et al., (2002) ; Umemori et al., (2004)

1.3.5: FGFR expression

In the earliest stages of the normal human developing skull and before ossification, FGFR1 expression is present in the entire head mesenchyme. FGFR2 is expressed in the epidermis and preosteogenic mesenchymal condensation and low levels of FGFR3 are found in basal head mesenchyme and epidermis. Approximately half way through gestation, sutures appear at the periphery of osteogenic centres and FGFR1 and FGFR2 are expressed at high levels in the osteogenic fronts of the sutures where only low levels of FGFR3 are found, (Delezoide et al., 1998). Studies in mice by Rice et al., (2003), found

mRNA for FGFR1c was detected weakly in osteoblastic cells in the developing calvarial bones. FGFR2c transcripts were detected at high levels in differentiating osteoblasts at the sutural osteogenic fronts of the calvarial bones. FGFR2b transcripts were detected in the cranial base where FGFR3b and FGFR3c were also localised. FGFR4, which has not been found to be a source of mutations for craniosynostosis, was detected minimally in calvarial cartilage. These distribution patterns are important in explaining the link between FGFR mutations and phenotype expression of individual craniofacial syndromes (Britto et al., 2001).

1.3.6: Mutations in syndromic craniosynostosis

As previously described in this chapter, the severest forms of craniosynostosis are associated with abnormalities of the molecular genetics which are summarised in **Table1.5**. The recent discoveries of mutated genes that cause syndromic craniosynostosis, and the subsequent molecular investigations into these mutations, have greatly enhanced our understanding of the biological events that underlie them.

In 1993 a breakthrough came when the first mutation to be associated with craniosynostosis was found in the MSX2 gene (Jabs et al., 1993). Subsequently, mutations in various locations of the FGFR2 gene were found in children with a clinical diagnosis of Crouzon syndrome (Reardon et al., 1994). Since then Crouzon, Apert, Pfeiffer, Jackson Weiss and Beare-Stevenson syndromes have all been shown to arise as a result of mutations in FGFR2 (Jabs et al., 1994; Muenke et al., 1994; Wilkie et al., 1995,). Mutations in FGFR1 and FGFR3 can also occur in rarer forms of Pfeiffer syndrome and Crouzon patients respectively (Muenke et al., 1994; Schell et al., 1995; Pryzlepa et al., 1996). Muenke syndrome, the commonest and the mildest of the syndromic craniosynostoses is caused by a mutation of FGFR3 which is more commonly the site of mutations causing achondroplasia. To date, no causal mutations have been reported in FGFR4 (Gaudenz et al., 1998) or in FGFR5 (Kim et al., 2001). The similarity in clinical phenotype of cases with Apert syndrome, reflects its restricted genotype compared to that of the other syndromes including Crouzon, Jackson-Weiss and Pfeiffer which exhibit an overlapping clinical phenotype. It is noteworthy that Apert syndrome is associated with increasing paternal age (Moloney et al., 1996) and implicates the involvement of spermatogenesis in mutagenesis.

Mutations in TWIST, a basic helix-loop-helix transcription factor have been identified in individuals with Saethre-Chotzen syndrome (Howard et al., 1997; El Ghouzzi et al., 1997). The mechanism for mutated TWIST activity is not clearly understood, although most of the mutations result in a non-functional protein and are likely to result in haploinsufficiency (Wilkie, 1997). Yousfi et al., (2001), found that this produced altered osteoblast function in cultured human calvarial cells which may be linked to alteration of FGFR expression (Rice et al., 2000; Funato et al., 2001). This has been confirmed by Guenou et al., (2005), who have demonstrated that TWIST haploinsufficiency downregulates Fgfr2 mRNA expression, which in turn reduces Runx2 and downstream osteoblast-specific genes in human calvarial osteoblasts. Thus, providing evidence for a role of FGFR2 in the altered osteoblast phenotype induced by TWIST mutations in Saethre-Chotzen syndrome. More recently, Connemey et al., (2008), have shown that TWIST happloinsufficiency alters the TWIST heterodimers/homodimer ratio, effecting sutural patency by altering suture responsiveness to FGF signalling. In contrast to the majority of mutations in the TWIST gene, all FGFR mutations are in-frame mutations which code for functional proteins and are due to missense mutations, deletions, insertions or splice events. Although many mutations have been reported, there is marked clustering of mutations at particular locations in the FGFR genes. Namely, at the interlinker region between IgII and IgIII, the membrane end of the 3rd immunoglobulin domain and the transmembrane domain (Wilkie, 1997). Homologous mutations have been identified at equivalent positions in more than one FGFR. For example, in the interlinker region between IgII and IgIII, Pro252Arg substitution of FGFR1 causes Pfeiffer syndrome, Pro253Arg substitution in FGFR2 causes Apert syndrome, and Pro250Arg substitution in FGFR3 causes Muenke craniosynostosis (Muenke et al., 1994; Wilkie et al.,, 1995; and Bellus et al., 1996). Interestingly, it has also been identified that single mutations such as the C278F missence mutation of the FGFR2, can manifest as either Crouzon, Pfeiffer or Jackson-Weiss syndrome (Meyers et al., 1996; Passos-Bueno et al., 1997). Moreover, the same syndrome can be caused by mutations in different genes as exemplified by mutations in both FGFR1 and FGFR2 causing Pfeiffer syndrome. The mechanism by which the same genotypes cause different phenotypes is still uncertain. It may be due to unidentified polymorphisms within FGFRs or a complex temporal interaction between receptor regulation and ligand availability within different domains (Wilkie et al., 2006).

Gene	Syndrome	Typical mutation sites/regions
FGFR1	Pfeiffer	IgII - IgIII interlinker
FGFR2	Crouzon	Ig III – mainly the creation of unpaired cysteine residues
	Pfeiffer	As for Crouzon, sometimes the mutations are identical (e.g. C278F)
	Apert	IgII-IgIII interlinker
	Jackson-Weiss	As for Crouzon
	Beare Stevenson	Creation of new cysteine residues
FGFR3	Muenke syndrome	IgII-IgIII interlinker (P250R)
	Achondroplasia	Transmembrane
	Crouzon (with a.n.)	Transmembrane
	Thanotophoric dysplasia I	Creation of cysteine residue
	Thanatophoric dysplasia II	Tyrosine kinase domain

Table 1.5: Craniofacial syndromes associated with mutations in FGFRs
Abbreviations: **Ig:** Immunoglobulin, **a.n.:** acanthosis nigricans. (Adapted from Wilkie, 2005)

There is evidence that mutations cause constitutive activation of receptors (Neilson & Friesel, 1995; Galvin et al., 1996; Mangasarian et al., 1997; Robertson et al., 1998). This activation is brought about by a number of effects including a) alteration in the number of cysteine residues allowing covalent cross linking with another mutant receptor and receptor dimerisation in the absence of FGF (Kannan and Givol, 2000), b) destabilisation of the disulphide bridge leading to alternative intermolecular bonds (Burke et al., 1998), c) formation of transmembrane hydrogen bonds leading to conformational change and dimerisation (Webster and Donoghue, 1996) or d) increased ligand affinity of reduced ligand dissociation, leading to prolonged receptor signalling (Anderson et al., 1998). (**Fig. 1.8**)

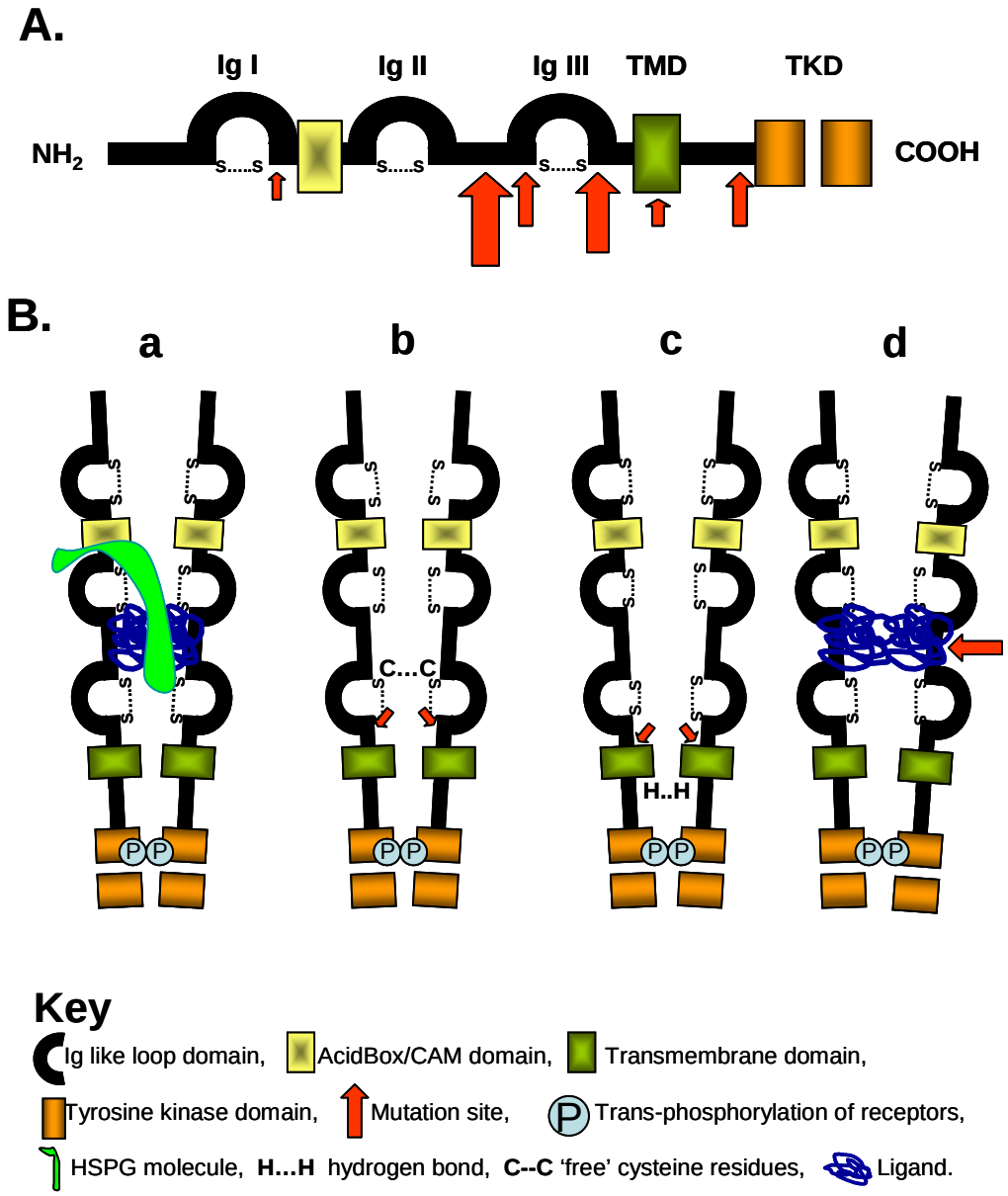


Figure 1.8: Schematic representation of FGFR mutations

A. General structure of FGFR showing mutation foci

B. Effects of mutations in syndromic craniosynostosis. a) normal FGFR activation, b) intermolecular disulphide bridge formation secondary to unpaired cysteines. c) hydrogen bond formation in transmenbrane domains d) mutations leading to increased affinity or reduced dissociation of FGF ligand. (Adapted from Anderson et al., 1998; Burke et al., 1998; Green et al., 1996; Kannan & Givol, 2000; Webster & Donohue, 1996)

1.3.7: FGF/FGFR interactions and osteogenisis

Bone development is dependent on the expression of members of the FGF family expressed locally during bone formation. FGF2, FGF4, FGF9, FGF18 and FGF20 transcripts are found in cranial and sutural mesenchymal cells and osteoblasts (Gonzalez et al., 1996; Kim et al., 1998; Britto et al., 1998; Rice et al., 2000; Ohbayashi et al., 2002; Hajihosseini and Heath, 2002), suggesting that they may be involved in regulating calvarial osteogenisis. Recent data indicates that FGF18 is becoming recognised as an important coordinator of osteogenisis via FGFR2 and FGFR3 and will be reviewed separately. FGF expression is also dependent on the temporo-spatial expression pattern of FGFRs (Powers et al., 2000). FGFR1 and FGFR2 are expressed in mesenchymal cells during condensation prior to deposition of bone matrix, at early stages of long bone development (Orr-Urtreger et al., 1991) and are also expressed in the cranial suture (Delezoide et al., 1998). Later in development and postnatally, FGFR1 and FGFR2 are found in pre-osteoblasts and osteoblasts together with FGFR3 (Iseki et al., 1997; Delezoide et al., 1998; Kim et al., 1998). The coordinate actions of FGFs on gene expression in osteoblasts is not only dependent on FGFR expression, but also on the affinity and specificity of FGF binding to the various alternative splice forms of FGFRs (Ornitz et al., 1996). FGFR2IIIc is expressed in early mesenchymal condensates and later in sites of endochondral and intramembranous ossification where it was recently found that in mice, the recessive phenotype of Fgfr2IIIc(−/−) is characterised initially by decreased expression of bone transcription factor RunX2 (previously Cbfa) and retarded long bone ossification, suggesting that Fgfr2IIIc is a positive regulator of ossification (Eswarakumar et al., 2002). Furthermore, FGFs bind cell surface heparan sulfate proteoglycans acting as low-affinity co-receptors that interact with FGF binding and signalling (Ornitz, 2000; Schlessinger, 2000). Some of these proteoglycans, including syndecans are expressed in bone (Solursh et al., 1990) and may affect the osteoblast response to FGF during osteogenisis (Molténi et al., 1999). Given this mix of interacting molecules, it is likely that subtle changes occurring in the expression or localisation of any number of them, may induce variable functional effects on FGF signalling and osteoblast gene expression in vivo. Moore et al., (2002), have shown that there is a gradient of FGF2 expression, which is greatest at the suture, lessening in the surrounding mesenchyme, suggesting FGF2 may be driving proliferation at the sutures to keep them patent.

This group further found that blocking FGF2 activity prevents osteogenisis in a concentration dependent manner, consistent with previous findings that over-expression of FGF2 in mice induces abnormal bone formation (Coffin et al., 1995), whereas FGF2

perturbation inhibits it (Montero et al., 2000; Greenwald et al., 2001). There is also good evidence that at the osteoblast level, the effect of FGFs is dependent on the stage of cellular maturation: and this will be discussed more fully in chapter 5.

1.3.8: FGF18 in osteogenisis

FGF18 is a recently investigated member of the FGFs, having been isolated in the rat and mouse in 1998 (Ohbayashi et al., 1998; Hu et al., 1998). The human FGF18 gene has been localized to chromosome 5q34 (Whitmore et al., 2000). It is highly conserved with a 99% identity match with the amino acid sequence, while the nucleotide sequence is 90% conserved between humans and mice. It is involved in specification of left-right asymmetry (Ohuchi et al., 2000) and in the development of the intestinal tract (Hu et al., 1998), brain (Ohuchi et al., 2000) and lung (Whitsett et al., 2002). A role in endochondral and intramembranous bone formation has been demonstrated in rodents and chicks (Ellsworth et al., 2002; Liu et al., 2002; Ohbayashi et al., 2002). Hajihosseini et al., (2002), found that FGF18 is strongly expressed in mouse coronal sutures, dorsal rims of the parietal bones and in the cranial base and that these regions directly abut with zones of osteopontin expression. It is interesting that of the FGFs so far targeted in mice, only those lacking FGF18 have demonstrated a phenotype affecting calvarial development: Ohbayashi et al., (2002) demonstrated FGF18 expression in differentiating osteoblasts and osteogenic mesenchyme, between the osteogenic fronts in calvarial bones and in the perichondrium of developing long bones. $FGF18^{-/-}$ mice illustrated delayed ossification characterised by widened metopic, coronal and sagittal sutures, delayed suture closure and decreased calvarial mineralisation. Proliferation within the calvarial osteogenic mesenchyme was also decreased.

FGF18 shows strong binding affinity for FGFR3c and FGFR2c, as well as strong mitogenic activity for FGFR3c, modest for FGFR2c and not at all for FGFR1, FGFR4 or any of the b spliced variants (Xu et al., 2000). Both $FGF18^{-/-}$ and $FGFR3^{-/-}$ mice display a similar although not identical long bone phenotype suggesting that while FGF18 signalling through FGFR3 is important in chondrogenisis, FGF18 must also signal through other FGFRs (Liu et al., 2002). More recently Liu et al., (2007), have emphasised the role of FGF18 in the control of mesenchymal vascularisation around centres of endochondral osteogenisis. Since FGFR1 and FGFR2 are the predominant FGFRs expressed in the calvarial bones (Iseki et al., 1999; Rice et al., 2000), it is likely that these FGFRs are the target for FGF18 in calvarial osteogenisis.

The MC3T3-E1 murine cell line treated with FGF18 show a dose dependent increase in proliferation through ERK activation and inhibition of matrix synthesis and differentiation. These effects were comparable to the effects produced by FGF2 and it has been postulated that it might compensate for FGF2 in skeletal development (Shimoaka et al., 2002).

1.3.9: FGF/FGFRs and osteoblast gene expression

The number of ligands and receptors acting on osteoblast are many and complex. However, FGF/FGFR interactions play an important part in modifying the expression of multiple genes that characterise the osteoblast phenotype. In osteoblastic cell cultures, as is the case for many other proteins, the effects of FGF signalling on osteocalcin (OC) expression are complex and depend on the cell type. Indeed, FGF1 inhibits OC gene expression in rat calvarial cells (Tang et al., 1996), but enhances its expression in bovine bone cells (Schedlich et al., 1994). Direct regulation has been reported in mouse calvarial cells, where FGF2 effects OC expression by acting on specific elements in the OC gene (Schedlich et al., 1994, Newberry et al., 1996), FGF2, FGF4 and FGF8, or transfection with a vector expressing a mutant FGFR2 that is constitutively activated in the presence of FGF ligand, was found to stimulate Runx2 expression in osteoblasts (Zhang et al., 2002; Kim et al., 2003). FGF signalling effects the expression of proteins involved in cellular communication and adhesion including the connexins and cadherins which will be discussed in more detail later in the chapter. FGFs interact with other growth factor signalling pathways and may thereby regulate osteoblast function. For example, FGF2 up-regulates TGFβ expression in osteoblasts in vitro (Noda & Vogel 1989). Conversely, TGFβ regulates FGF2 and FGFR expression in osteoblastic cells (Sobue et al., 2002). Additionally, FGF2 increases insulin-like growth factor-I (IGF-I) gene expression in vitro (Zhang et al., 2002) and in vivo (Power et al., 2002). Recent data indicate that FGF2 and FGFR2 inhibit the expression of the bone morphogenic protein (BMP) antagonist noggin in the patent cranial suture, resulting in premature suture fusion (Warren et al., 2003), thus indicating that FGF signling can control cranial suture fusion indirectly through BMP signalling. It is therefore likely that the biological activities of FGFs in bone depend not only on the balance between FGF and FGFR expression, but also on the presence of other signalling molecules. In addition, FGFs regulate genes that are involved in matrix resorption by increasing the expression of extracellular collagenase (Hurley et al., 1996, Varghese et al., 2000). These studies suggest that FGFs may modulate bone matrix breakdown by regulating collagenase expression and activity. Taken together, activation of

FGF/FGFR signalling seems to regulate genes involved in most of the steps of osteogenesis. These finding would also suggest that genes may participate in regulating cell progression from osteoprogenitor cell to osteoblast death

1.4: Cellular bone biology

1.4.1: Bone Cells

Bone is a constantly changing tissue which necessitates the presence of cells both for deposition and resorption. Calvarial bones ossify by the direct secretion of extracellular matrix, by osteoblasts, into mesenchymal condensations, forming centres of ossification. Osteoblasts are the predominant cell of bone deposition and are derived from mesenchymal cells which become preosteoblast prior to becoming functioning osteoblasts. Osteoclasts, which are derived from the monocyte/macropghe lineage, play an equally important role in bone growth and remodelling by their function of resorption (Sommerfeldt & Rubin 2001). They are regulated by the acidified microenvironment in addition to being under hormonal control by numerous factors, both pro-resorbative such as vitamin D3 and corticosteroid and anti-resorbative such as oestrogen and calcitonin (Boyle et al., 2003). Osteocytes are found, throughout mature bone enclosed within lacunae. They are the most common cell type in bone, forming an intercommunicating, syncitial network. Their role in osteogenisis and skeletal health has until recently been unclear. However, it is now appreciated that osteocytes are highly responsive to circulating hormone levels and cytokine (Bringhurst, 2002), and there is now growing evidence to support the hypothesis that the osteocyte is the bones mechanoreceptor (Noble et al., 2003).

1.4.2: Osteoblast differentiation and function

Osteoblasts are channelled through a path of commitment from mesenchyme to become mature osteoblasts. This journey from pluripotent mesenchymal cell to fully differentiated and functioning osteoblast is a process which involves a variety of hormones, cytokines and growth factors (Roelen, 2004). Osteoblasts are principally responsible for the deposition and regulation of extracellular matrix proteins and the expression of genes required for mineralisation and interaction with osteocytes and osteoclasts to regulate bone turnover. Molecules important for development of the osteoblastic phenotype include, Runx2 a transcription factor required for osteoblast differentiation and bone formation (Ducy et al., 1997). Absence of Runx2 in knock-out

mice causes a complete absence of osteoblasts and osteogenisis (Komori et al., 1997, Otto et al., 1997). In humans, mutations within the Runx2 gene cause cleidocranial dysplasia (Lee at al 1997; Mundlos et al., 1997), characterised by short stature, widely patent inter-parietal and metopic regions and dental anomalies.

Osterix (Osx) is a recently identified zinc-finger containing transcription factor, that is expressed in all developing bones and appears necessary for osteogenisis. *Osx* null mice, do not deposit bone matrix and membranous mesenchymal cells cannot differentiate into osteoblasts. These cells do, however, express Runx2. In contrast, Osx is not expressed in Runx2 null mice, suggesting that Osx acts downstream of Runx2 (Nakashima et al., 2002).

Mature osteoblasts are morphologically indistinguishable. However recent in-situ studies in rat calvaria show that they are not a homogeneous class of cells, but are instead a molecularly diverse group of subpopulations based on the expression patterns of specific markers. These can be divided into two categories; those that are expressed by all osteoblasts, irrespective of their position in the calvaria, including, alkaline phosphatase and those such as the matrix proteins osteopontin, osteocalcin and bone sialoprotein which are differentially expressed only in subpopulations of osteoblasts (Candeliere et al., 2001). This work complements previous, similar findings of osteoblast subpopulations within bone nodules of cultured rat osteoblasts (Malaval et al., 1994). The mRNA expression of some of the important markers of osteogenisis will be studied in this work.

1.4.2.1: Type 1 Collagen

Synthesised by fibroblasts and odontoblasts, type 1 collagen is principally the product of osteoblasts during the laying down of bone matrix and is the most abundant protein in bone, accounting for approximately 90% of bone matrix (Bilezikian et al., 1996). It is a trimeric molecule composed of two α1 chains and a α2 chain, which are in themselves made from polypeptide sequence repeats. These allow the formation of a triple helix which gives rise to a highly ordered fibrillar structure, important for tensile strength within mineralised tissues. Mutations within the genes coding for collagen I α chains lead to osteogenisis imperfecta, characterised by fragility and deformities of bone and dentine (Penttinen et al., 1975). Ascorbic acid is required for normal fibrillar crosslinking, which in turn is necessary for expression of the osteoblast phenotype and expression of alkaline phosphatase, osteocalcin (Franceschi et al., 1994; Wenstrup, 1996; Xiao et al., 2002) and for optimal cell adhesion and migration (Masi et al., 1992). Studies indicate that interaction of type I collagen with alpha2 integrin, is a crucial signal for the induction of osteoblastic differentiation and matrix mineralisation (Xiao et al., 1998; Reyes et al., 2004)

Pathologically increased secretion of type 1 collagen by calvarial osteoblasts has been demonstrated in craniosynostosis associated with mutations of FGFRs (Bodo et al., 1996; Lomri et al., 1998; Baroni et al., 2002).

1.4.2.2: Alkaline Phosophatase

Alkaline phosphatase (ALP) is a glycoprotein phosphohydrolase which exists in four isomeric forms encoded by different genes. The ALP associated with bone metabolism is tissue non-specific. This is found as a membrane bound enzyme in many tissues including some preosteoblasts and all osteoblasts (Candeliere et al., 2001). The function of ALP in bone formation is not entirely clear. However, it is thought to play a role in mineral maturation, as humans carrying mutations for ALP suffer from variable degrees of hypophosphatasia, a condition characterised by poor bone mineralisation (Weiss et al., 1988). ALP is an early marker of the osteoblast (Whyte et al., 1994) phenotype, with expression which is inversely related to cellular proliferation increasing as cellular maturation progresses (Fedarko et al., 1990; Cowls et al., 1998).

Alkaline phosphatase activity requires normal type 1 collagen secretion (Wenstrup et al., 1996) and decreased levels of ALP have been demonstrated in the calvarial osteoblasts of patients with craniosynostosis associated with Crouzon syndrome (Ratisoontorn, 2003).

1.4.2.3: Osteocalcin

Osteocalcin is a highly conserved, noncollagenous matrix protein found in bone, and other mineralised tissues (Ulrich et al., 1987) and is amongst the most abundant of the bone matrix proteins. (Gerstenfeld et al., 1987). The functions of osteocalcin, suggested by an analysis of genetically engineered osteocalcin-deficient (“knockout”) mice includes, bone mineral maturation and inhibition of bone formation. Osteocalcin knockout mice have an accelerated rate of bone formation without changes in osteoblast or osteoclast number (Ducy et al., 1996). It is highly expressed by mature osteoblasts, usually as a late marker of osteoblast differentiation and is commonly examined in studies of osteogenisis. Raised OC levels have been demonstrated in craniosynostosis associated with mutations in the FGFRs (Lemmonier et al., 2001; Tanomoto et al., 2004) whilst decreased levels have been expressed in other craniosynostoses, associated with mutations in the TWIST gene (Yousfi et al., 2001).

1.4.3: Coordinate expression profile of osteodifferentiation markers

Osteoblastic cell cultures, both primary and cell line, demonstrate a coordinate pattern of gene expression for molecules associated with the osteoblast lineage. The pattern of expression is dependent on the cells progressing through recognised stages from proliferation to terminal differentiation usually leading to mineralisation. Generally, the differentiation sequence is defined by an increase in collagen synthesis, acquisition and increase in alkaline phosphatase activity, followed by expression of several non-collagenous bone matrix proteins, such as osteopontin, bone sialoprotein and finally osteocalcin as the latest marker of the mature osteoblast (Quarles et al., 1992; Heersche et al., 1992; Choi et al., 1996). However, discrepancies in this expression profile have been reported and it should be borne in mind that whilst the general pattern exists across species, expression patterns are species specific; for example osteocalcin is expressed at a later stage of differentiation than ALP in the rat calvaria, whereas it parallels expression of ALP in chick calvaria (Stein & Lian 1993; Gersternfeld et al., 1987). Within osteoblast lines, expression of different levels of the same marker are even observed (Heersche et al., 1992; Malaval et al., 1994; Smith et al., 2000), indicating considerable plasticity and functional heterogeneity of the osteoblast phenotype; for example within the MC3T3-E1 osteoblast line which shall be examined more fully later, subclones of cells were identified in which expression of ALP was highly variable (Wang et al., 1999).

1.4.4: Cell adhesion and communication

As well as regulating the expression of genes characterising the osteoblast phenotype, FGF/FGFR signalling controls osteogenisis indirectly, via the expression of genes involved in osteoblast function, including, amongst others, those of cell adhesion and communication channel proteins. These are required for cell-cell and cell-matrix interaction, communication and adhesion (Francheschi, 1999).

1.4.4.1: N-Cadherin

Cell-cell adhesions are mediated by adherens junctions containing cadherins. These are transmembrane glycoproteins acting with intracellular partners known as catenins which in turn interact with intracellular proteins (Yamada & Geiger, 1997). The cadherin family is composed of several calcium dependent molecules of which N-cadherin is one. Human osteoblasts were first shown to express N-cadherin by Cheng et al., (1998), who

demonstrated that cadherins were critical for the expression of Bone Morphogenic Protein (BMP)2.

During the early stages of intramembranous bone formation, cadherins play an important role in the condensation of mesenchymal cells prior to ossification (Hall & Miyake, 2000). Osteoblasts in human and mouse cranial sutures and preosteoblasts and osteoblasts in the foetal human cranial sutures are known to express N-cadherin (Tsutsumimoto et al., 1999; Lemmonier et al., 2000). In human calvarial osteoblasts, N-cadherin neutralising antibodies have been shown to down-regulate the expression of alkaline phosphatase, type I collagen and osteocalcin (Hey et al., 2000). Lemmonier et al., (2001) and Lomri et al., (2001), have demonstrated over-expression of N-cadherin, mediated by Protein Kinase C, in osteoblasts from the coronal suture of fetuses with Apert syndrome carrying an activating mutation for FGFR2. This resulted in increased cell-cell adhesion and over-expression of osteodifferentiation markers in days 0-7 of confluent primary cell cultures.

1.4.4.2: Connexin 43

Intercellular communication is a fundamental requirement of skeletal tissue and is thought to control several physiological functions of bone cells, including, the coordination of tissue responses to mechanical stimulations (Duncan & Turner, 1995), sensitivity to hormones and growth factors (van der Molen et al., 1996) and the progression of differentiation (Lecanda et al., 1998). This communication is mediated by the transfer of small molecules and ions through transmembrane channels called gap junctions which are abundant in bone cells (Stanka, 1975; Doty, 1981) and have been demonstrated in cultured osteoblasts including MC3T3-E1 cells (Yamaguchi et al., 1994).The principal functional components of gap junctions are the connexins. These comprise a closely related group of hexameric proteins that associate to form cell-cell adhesion channels which assemble in gap junctions (Makowski et al., 1977; Beyer et al., 1990). When the connexin of one cell is in contact with a similar structure on a neighbouring cell, a transcellular channel is formed (Civitelli, 1995). The most abundant connexin in human and MC3T3-E1 osteoblasts is connexin 43(Cx43) (Civatelli et al., 1993; Yamaguchi et al., 1994). The gene is located on human chromosome 6q22. Mutations within the gene cause Oculo-digital-dental dysplasia, characterised by ocular, nasal, limb and dental deformities (Paznekas et al., 2003). The expression of Cx43 is associated with elevated levels of metabolic coupling (Jorgensen et al., 1997) and with a high degree of cellular differentiation (Schiller et al., 1997; Li et al., 1999). Over-expression of Cx43 has resulted in

increased expression of osteocalcin in MC3T3-E1 cells (Lecanda et al., 1998), whilst Cx43-null mice display globally delayed ossification, osteoblast dysfunction and craniofacial abnormalities characterised by poor membranous bone ossification (Lecanda et al., 2000). Cx43 has also been implicated in altered cell motility and adhesion; Xu et al., (2001), have reported that neural crest cells deficient in Cx43 displayed poor motility and delayed neural crest migration has been observed by Huang et al., (1998). Gramsch et al., (2001), reported enhanced proliferation, bone nodule formation and mineralisation of the UMR osteosarcoma cell line overexpressing Cx43. FGF2 treatment has been shown to down-regulate Cx43 dependent cellular communication in astroglial cells (Reuss et al., 1998) and in MC3T3-E1 cells (Shiokawa-Sawada et al., 1997).

1.5: Bone engineering

1.5.1: The "Ideal" biomaterial

There is a strong need for bone substitution materials in craniofacial reconstruction and surgery in general. Currently, standard autologous bone grafting remains the default "best option" for bony reconstruction of the craniofacial region, although frequently this is not feasible and several allograft options, along with their potential problems, do exist. However, primary bone grafting fails in up to 20% of cases for a number of reasons (Younger & Chapman, 1989) and harvesting requires a donor area often at a secondary site. Thus, in addition to potentially increasing the operative time, this also increases the potential complications as reviewed in section 1.2.6.1. It would be desirable therefore, to try and eliminate these associated problems by creation of a bone graft substitute. An engineered bone substitute that can mimic the native structure and replace the mechanical and biological functions of the natural tissue being replaced would be ideal. This "ideal" would be a mechanically appropriate, biocompatible, bioresorbable scaffold, capable of supporting sufficient numbers of functioning cells (Langer & Vacanti, 1993), capable of regenerating bone.

The search for this ideal synthetic bone substitute has been long and has generated numerous and ongoing studies (Rose & Oreffo, 2002). Initial attempts to produce materials with this aim, had as their main objective, the provision of implants which were physically suitable and which did not involve unacceptable host toxicity. This principally meant materials which were inert and did not produce a foreign body reaction. This resulted in more than 50 prosthestic implants being introduced into clinical use in the

1960s-70s. None of which however, attempted to allow or induce bone regeneration (Hench, 1980).

More recently, work has focused on the two material properties which seem fundamental to creation of the “ideal”. Firstly, the creation of biodegradable materials and secondly the creation of materials which either permit bone regeneration by supporting osteoblast growth (*osteo**con**ductive*), or actively stimulate bone regeneration by inducing cells to differentiate down the osteoblast lineage to form bone (*osteo**in**ductive*). The classic example of the latter being the induction of ectopic bone within muscle following the intramuscular injection of demineralised bone matrix (DBM) (Urist, 1965).

Biodegradable materials offer several physical advantages over metals and other non-degradable materials currently in use as orthopaedic or craniomaxillofacial implants. These are best described in terms of avoiding the reported disadvantages inherent to non degradable materials such as titanium. These include growth disturbance (Resnick et al., 1990; Lin et al., 1991; Eppley et al., 1993), plate migration (Fearon et al., 1995), the need for subsequent removal (Beals & Munro, 1987; Schmidt & Perrot, 1998) compatibility with future imaging needs, long-term palpability and thermal sensitivity (Orringer et al., 1998).

Numerous naturally occurring, biodegradable compounds have been studied in an attempt to create scaffolds including collagen (Mizuno et al., 1997), chitosan (Lee et al., 2000), alginate (Shang et al., 2001), agarose (Mauck et al., 2003) and fibrin (Karp et al., 2004). While these are degradable and biologically derived, their utility has been questioned on the basis that they provide poor mechanical strength and degradation rates can prove inconsistent (Gombotz et al., 1993). Synthetic alternatives which provide mechanical strength have therefore been studied and developed, including polycarbonates, polyesters, polyorthoesters, polyanhydrides, polyurethanes and polyphophosphazenes (Gunatillake & Adhikari, 2003). As a result of these developments, several have been licensed for clinical use.

1.5.2 Biomaterial options

1.5.2.1 Polyesters

One of the most extensively studied biodegradable, polymeric materials, are the group known as the polyesters, of which polylactic acid (PLA) and polyglycolic acid (PGA) are the two most commonly encountered. They have been used for a number of clinical applications including resorbable sutures, drug delivery systems and fixation devices such as rods, pins screws and plates (Ashammakhi & Rokkanen, 1997, Middleton & Tipton, 2000). They have also been studied for tissue engineering applications (Hollinger, 1983; Wong & Mooney, 1997), particularly bone engineering (Ishaug-Riley et al., 1998; Burg et al., 2000).

1.5.2.2 Polylactic acid (PLA, L-PLA)

Polylactic acid is a naturally occurring compound produced from readily renewable recourses such as corn and sugarbeet, which when polymerised to form polylactic acid is used in a wide range of industrial applications where natural degradation is a requirement (Drumright et al., 2000). Polylactic acid exists as two optical isomers, a poly-L-lactide and a poly-D-lactide. The poly-L isomer is a semicrystalline polymer with a high melting point (178^{o}C) and glass transition temperature (65^{o}C). These properties impart high tensile strength and a prolonged degradation time (3-5 years) to materials constructed from it.

There are few reports of the use of L-PLA, although successful outcomes have been reported in craniomaxillofacial surgery (Bos et al., 1987, Waris et al., 1994). However the long degradation time has been associated with chronic foreign body tissue reactions to PLA crystal remnants (Bergsma et al., 1993). Thus the long degradation time appears to have negated the advantages of a resorbable material. However, polymerisation of a poly-D, poly-L isomer blend (PLA), gives rise to an amorphous lower melting point (60^{o}C), weaker compound with a correspondingly shorter degradation time (12-16months) (Gilding & Reed, 1979). This blend gives rise to a degradation profile which theoretically should be less likely to cause chronic reactions. Haers & Sailer, (1998) and Turvey et al., (2002), have reported 10 and 55 cases respectively with good mandibular and maxillary fixation with minimal complications following osteotomies. Serlo et al., (2001), have used PLA plates and screws in 15 children undergoing cranioplasties and reported good clinical results with no complications at 8 year follow-up.

1.5.2.3 Polyglycolic acid (PGA)

Like PLA, polyglycolic acid is formed by polymerisation of glycolic acid to produce a semicrystalline structure with a melting point reported to be in the range of 225-230°C and a glass transition temperature between 35-40°C. There are no isomeric forms (Gilding & Reed, 1979). PGA has the greatest flexural strength of the polyesters but undergoes the most rapid degradation with the majority of its strength lost by 6 weeks and complete volume loss by approximately 9 months (Imola et al., 2001). A major medical application of PGA came in 1970 with its introduction as a braided, resorbable suture known as Dexon (American Cynamide Company), (Bender & Brouwer, 1975), which is still in current use, testifying to its long term safety and biocompatibility. However, given the relatively rapid degradation of PGA, its clinical use as a single constituent for the manufacture of rigid or semi-rigid fixation devices has not been reported, although their successful use as a "membrane" for supporting orbital contents in orbital floor repairs, where minimal mechanical loading is involved, has been reported (McVicar et al., 1995).

1.5.2.4 Polylactic-co-glycolic acid copolymers (PLGA)

Copolymerisation of PLA and PGA disrupts the crystallinity of these monomers, leading to an amorphous network with a degradation rate dictated by the monomer ratio. The ratio of monomers used in the copolymerisation process allows the physical properties of the polymer to be manipulated, which in turn alters the degradation properties of the polymer (Gopferich, 1996). The first commercial use of this copolymer range was in 1971 with the resorbable suture material Vicryl (Ethicon inc.) composed of 8% PLA and 92% PGA (Aston & Rees, 1976).

Commercially available fixation devices blend approximatelly 80% PLA with 20% PGA, to reflect the combination of degradation and structural strength required. The copolymer is largely amorphous and has a degradation profile between PLA and PGA Clinically, It has been reported as providing successful rigid bony fixation, retaining 70% of its initial strength for 6-8 weeks and has disappeared between 12-16 months after implantation (Tatum et al., 1997; Peltoniemi et al., 2002). Eppley et al., (2004), retrospectivelly reviewed a multicenter experience of 1883 cases of craniosynostosis in which PLGA plates/screws were used. Significant infectious complications occurred in 0.2 percent, device instability primarily resulting from postoperative trauma occurred in 0.3 percent, and self-limiting local foreign-body reactions occurred in 0.7 percent of the treated patients. The overall re-operation rate attributable to identifiable device-related problems was 0.3 percent. A further retrospective multicentre study of 165 cases (Ashammakhi et

al., 2004) stated that PLGA plates were safe and effective although noted an overall complication rate of 7.2% not all of which were directly attributable to the fixation device.

The degradation process takes place in two steps during which the polymer or copolymer is converted back to its monomer acids. Firstly tissue fluid diffuses into (non-crystalline) regions of the polymer matrix, cleaving the ester bonds; the second step starts after the amorphous regions have been eroded, leaving the crystalline portion of the polymer susceptible to hydrolytic attack by random hydrolysis and tissue esterases. The degradation product, glycolic acid, is non toxic and this then excreted directly in urine or enters the citric acid cycle after which it is excreted as water and carbon dioxide. (Drumright et al., 2000).

1.5.3 Bioactive glasses

The bioactive glasses are a group of biodegradable and biocompatible materials, originally developed by Hench in the 1960s (Hench et al., 1971). The structure which is manufactured in several formulations, depending on the percentage of SiO_2, consists of silica, calcium, sodium and phosphate. This develops a hydroxyapatite layer when exposed to tissue fluids, which has been shown to be osteoconductive (Hench et al., 1971). More recently, Yuan et al., (2001), have demonstrated the limited osteoinduction of ectopic bone in the muscle of dogs implanted with bioactive glass.

Bioactive glasses have been shown in microarray analysis of primary human osteoblasts to activate genes associated with bone formation within a few hours of exposure to bioactive glass dissolution products in a concentration dependant manner (Xynos et al., 2001). Bielby et al., (2004), have shown that murine embryonal stem cells cultured in the presence of bioactive glass dissolution products will develop down the osteoblast lineage to express markers of bone differentiation and form mineralised bone nodules.

Although glasses demonstrate good compressive strength, they are very brittle and have poor tensile strength. This makes them unsuitable for fixation purposes but has shown them to be effective in clinical applications where tensile forces are absent or minimal including grafting of periodontonal bone defects (Larmas et al., 1995), filling of frontal sinuses (Peltola et al., 1998), reconstruction of orbital floor fractures (Aitasalo et al., 2001) and maxillary sinus augmentation (Turunen et al., 2004).

In an attempt to utilse a strengthened formulation of bioactive glass for bone tissue engineering, work in our laboratory has shown that a composite scaffold consisting of bioactive glass and PLGA co-polymer, supports growth and progression of bone

differentiation of cell lines and primary osteoblasts carrying mutations for craniosynostosis. (Santos-Ruiz et al., 2007).

1.5.4 Bio-material composites

The ability of materials to induce significant regeneration of new bone appears from the literature to be limited. Hence the hypothesis that cells on polymer scaffolds could give rise to organised tissue was developed from the observations of Vacanti, (1988) that; a) all tissues undergo constant remodelling, b) dissociated mature cells can reorganise themselves into their native histological structure when placed in appropriate culture conditions, c) this reorganisation is limited by lack of a template to guide restructuring and d) the volume of tissue that can be implanted is restricted by gas and nutrient requirements.

Material scaffolds have to date principally acted as carriers and support structures on which osteoblasts must be able to survive and produce bone, although as will be discussed later, strategies are being developed to enhance this interaction. In this bio-composite approach, a scaffold is seeded with osteogenic cells. The interaction which ensues and subsequent success of the approach, will be dependent upon the chemical and surface properties of the material, the cell type, and the implant environment.

1.5.5 Surface and chemical properties

The primary interaction between cell and material, involving attachment and spreading is likely to be highly influential in further proliferation and differentiation and is therefore of great importance. The initial short term adhesion will involve physical forces such as ionic and van der Walls forces, followed by longer term molecular interactions including the material surface, ECM proteins and cytoskeleton proteins (Anselme et al., 2000) which are likely to act in concert to induce signal transduction to ultimately regulate gene expression. Initial interaction and subsequent cellular growth is affected by levels of surface free energy of different materials (Schakenraad et al., 1986) and also by differing ionic charges between cell types which affects the ability of cells to attach and spread on solid substrata (Schakenraad et al., 1988). In addition, cells do not interact with bare materials either in-vivo or in-vitro, and surface energy and charge effects the adsorption and structural arrangement of tissue fluid proteins on a material (Chesmel et al., 1995; Boyan et al., 1996; Shelton et al., 1998). The cellular adhesion properties of fibronrctin (FN) were altered by the surface properties of materials, affecting the ability of endothelial cells and fibroblasts to spread and adhere (Iuliano et al., 1993; Altankov et al., 1997).

El-Amin et al., (2003), have demonstrated that PLGA surfaces are more hydrophilic than a PLA surface and that human osteoblast adhesion to PLGA was significantly higher than on PLA surfaces. However, this difference became less marked as incubation time increased post-plating, concluding that cellular adhesion was also dependent on either the chemical composition that differs between PLGA and PLA, or the accumulation of degradation products including lactic acid. These authors also found that cells grown on PLGA produced higher levels of ECM molecules and cytoskeletal organisation than seen on PLA or tissue culture plastic. It is possible to surmise from these findings that the different formulations produce varying concentrations of breakdown products which may differentially effect cellular function.

The surface roughness of materials also causes cells to react differently. Osteoblasts have been shown to spread and form continuous layers more readily on smooth surfaces (Naji & Harmand, 1990; Anselme et al., 2000). A smooth surface also appears to benefit proliferation of osteoblasts with appropriately decreased levels of OC and ALP when grown on hydroxyapatite or titanium surfaces (DeSantis et al., 1996)

1.5.6 Scaffold modification

Each biomaterial has its own characteristic advantages and disadvantages. For this reason and in an attempt to optimise physical and regenerative capabilities, workers in the field have sought to combine properties by producing composite materials. Indeed, natural bone matrix is an organic-inorganic composite of collagen, matrix proteins and apatites and it could be postulated that composite materials are the obvious choice for bone engineering. For example, in an attempt to make glasses structurally more suitable for bone engineering, material composites of bioactive glasses or apatites and polyesters have been developed (Kikuchi et al., 1997; Marcolongo et al., 1998; Kellomaki et al., 2000). Moreover, it has been discovered that the alkaline dissolution products of glasses and the acidic degradation products of polyesters, can neutralise each other to create a physiological environment for optimised cell survival (Lu et al., 2003)

The three dimensional (3-D) structure of scaffolds has been considered to be an important part of scaffold design for bone engineering (Yamamoto et al., 1997; Griffith 2002; Hutmacher, 2000). The scaffold should have a high porosity and interpore connectivity, to allow a large number of cells to penetrate the scaffold and to allow the efficient diffusion of nutrients and metabolites to and from the cells (Vacanti et al., 1988). This has led to the development of numerous scaffold forming techniques including solvent casting and particulate leaching (Lu & Mikos 1994; Ma & Choi, 2001), gas foaming (Harris

et al., 1998), freeze-drying (Whang et al., 1995) electro-spinning (Renejer & Chun, 1996; Boland et al., 2001; Yoshimoto et al., 2003), phase-separation (Nam & Park, 1999) and rapid-prototyping (Landers et al., 2002).

Further innovative methods of formulating and producing scaffolds with variable physical characteristics have been described, which can for example successfully trap and release osteogenic enhancers, principally recombinant proteins, such as BMP-2 and TGF-ß1 (Tielinen et al., 1999; Oldham et al., 2000). Recently, pre-osteoblasts have been grown on scaffolds which have incorporated DNA plasmids on PLA based, electrospun nano-fibers, promising a flexible, biodegradable carrier capable of transfecting adjacent cells (Shea et al., 1999). Materials incorporating antibiotics have been reported (Overbeck et al., 1995; Zhang & Zhang, 2002); of particular potential benefit, carriers of antibiotics against Staphylococcal aureus species, the species having the greatest infective risk for implanted materials (Kim et al., 2004). However, difficulties have been reported when incorporating pharmaceuticals or co-materials. These stem from alterations to the material properties of the carrier, including weakening of constructs upon addition of antibiotics and the addition of bioactive glasses to polyesters (Tianen et al., 2002, Leinonen et al., 2002).

The surface nature of a material can directly affect cellular attachment, proliferation and phenotype, which ultimately will affect the ability of a system to regenerate new bone (Yamamoto et al., 1997). For example, polyesters are poorly hydrophilic, which greatly effects cell adhesion onto the surface and penetration into the scaffolds (Mikos et al., 1994). Furthermore, there are no cell recognition sites on the
surfaces of most tissue scaffolds which leads to poor cell affinity and potentially failed tissue engineering. (Liu et al., 2004). In recognition of this, surface modification is an area which has received considerable attention in the quest to optimise osteogenisis. This can be achieved by physico-chemical modification of the material surface or coating of the material.

1.5.7 Substrates adhesion

Cell-protein-surface interactions are critical to the development of biotechnological applications including biomaterials and tissue engineering. In many of these applications, cells interact with proteins synthesised and secreted by cells or adsorbed from physiological tissue fluid. Cellular interaction with ECM constituents provides signals that control cell survival, proliferation and phenotypic expression (Werb et al., 1989; Adams & Watt, 1990). Therefore, extracellular matrix components are likely to be important in regulating the survival and function of osteoblasts (Meredith & Schwartz, 1997), **(Fig 1.9).**

Osteoblast differentiation is partially dependent on adhesion to bone matrix proteins such as type1 collagen, laminin and fibronectin (Damsky, 1999; Franceschi, 1999; Zimmerman, 2000). These interactions between matrix proteins and osteoblasts are mediated in part by a group of transmembrane glycoproteins known as integrins (Plow et al., 2000) and more specifically the ß1 integrin subunits (Gronthos et al., 2001), which have been shown to mediate early osteoblast differentiation (Moursi et al., 1997) and which represent the primary mechanism of cell-ECM interaction across all cell types. (Hynes, 1992). Many bone ECM proteins including FN and laminin have chemotactic or adhesive properties, notably because they contain an Arg–Gly–Asp (RGD) sequence which is specific to the fixation of cell membrane receptors such as integrins which contain RGD recognition domains (Takada, 1997). Modification of PLA films by attaching RGD peptide to the surface has led to improved cellular attachment and spreading (Cook et al., 1997; Quirk et al., 2001).

1.5.7.1 Fibronectin

Fibronectin is widely expressed by multiple cell types and is critically important in vertebrate development, as demonstrated by the early embryonic lethality of mice with targeted inactivation of the FN gene (George et al., 1993). Although FN molecules are the product of a single gene on chromosome 2, the resulting protein can exist in multiple forms that arise from alternative splicing of a single pre-mRNA that can generate as many as 20 variants in humans (Kosmehl et al., 1996) which are tissue and function dependent (Xia & Culp, 1994; Sinkin et al., 1995; Price et al., 1998). It is a large dimeric ECM glycoprotein which has been shown to regulate adhesion, migration and differentiation of an array of mesenchymal cells (Yamada et al., 1992).

The FN molecule is a dimer of similar or identical subunits crosslinked by a pair of carboxyl-terminal disulphide bonds. Fibronectin interacts with cell surface receptors and other ECM components via well defined domains. The amino-terminal domain of FN binds to collagen, while the carboxy-terminal domains and central cell binding domains which include the Arg–Gly–Asp (RGD) sequence, have sites for interaction with cell surface receptors, including selectins, cadherins, immunolgobulins and integrins (Ruhoslahti & Pierschbacher, 1987, Damsky & Werb 1992; Hynes, 1992).

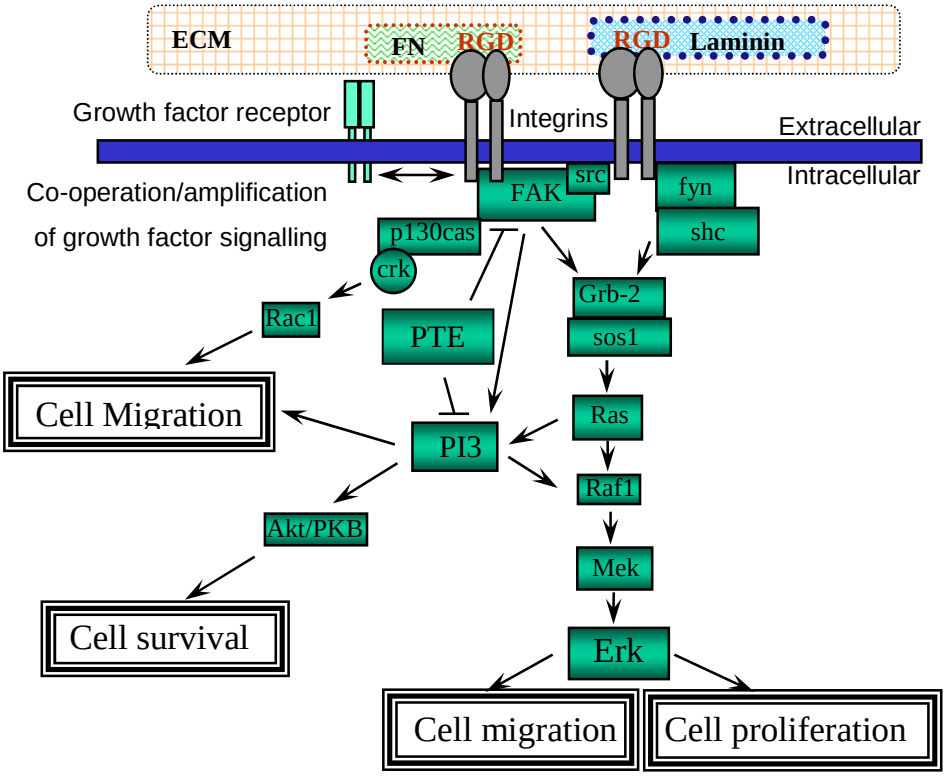


Figure 1.9: Interaction of extracellular matrix protein with intracellular signalling and cell functions via membrane receptors.

This diagram schematically represents the role of ECM protein binding and the influence of downstream effects via multiple signalling pathways (adapted from van der Flier and Sonnenberg 2001, Lee & Juliano, 2004)

Fibronectin is a main ligand for integrins and specifically the α5ß1 subunit which only binds FN (Werhle-Haller & Imhof, 2003). Varner et al., (1995), demonstrated that human colonic cells overexpressing integrin α5ß1 showed growth arrest which could be reversed by ligation of this receptor with fibronectin. Activation of the intracellular FAK and ERK2 signalling pathways in CHO cells is directly proportional to the level of FN-integrin binding (Asthagiri et al., l999). This has been confirmed by Carvalho et al., (2003), who

demonstrated that enhanced adherence of chicken calvarial osteoblasts to FN, stimulated FAK translocation from the nucleus to the cytoplasm, with a concomitant increase in expression of osteopontin. Moursi et al., (1996), showed that the addition of anti-fibronectin antibodies to cultures of osteoblasts from fetal rat calvaria at confluence reduced subsequent formation of mineralised nodules to less than 10% of control values and suppressed the expression of osteocalcin and alkaline phosphatase. The same authors subsequently showed that this effect was mediated through a decrease in fibronectin-integrin interaction (Moursi et al., 1997). Yang et al., (2001), demonstrated increased attachment, spreading and differentiation of osteoprogenitors derived from human bone marrow cultured on PLGA scaffolds and PLA membranes treated with FN and the RGD peptide. The attachment and proliferation of MC3T3-E1 osteoblasts onto polystyrene (Stephansson et al., 2002) and dimethylsiloxane, a synthetic polymer (Toworfe et al., 2004) has been enhanced by FN coating. Moreover, it has been shown that disrupting the osteoblast-fibronectin interaction can induce massive apoptosis in differentiated osteoblasts (Globus et al., 1998). It is therefore apparent that FN is crucial for expression of the osteoblast phenotype and that cellular attachment, proliferation and differentiation might be the subject of FN manipulation.

1.5.7.2 Laminin

Laminins are a family of glycoproteins that make up an integral part of the structural scaffolding of basement membranes in almost every animal tissue. Each laminin is a heterotrimer of which twelve exist in mammals, assembled from α, ß and γ chain subunits, which are secreted and incorporated into cell-associated extracellular matrix (Sasaki et al., 1988). Like fibronectin, the subunit chains can be alteranively spliced to produce multiple isoforms (Colognato & Yurchenco, 2000). Laminins bind to other matrix macromolecules, and have unique and shared cell interactions mediated by integrins, and other receptors (Henry et al., 2001). Through these interactions, laminins critically contribute to cell differentiation, cell shape and movement, maintenance of tissue phenotypes, and promotion of tissue survival. Targeted disruption of laminins causes a wide spectrum of developmental faults depending on subunit specificity, from early embryonic lethality with defects in placental vessels, anterior neural tube closure, kidney and limb development (Miner & Campbell, 2002) to central and peripheral nerve defects, muscular dystrophies (Sunada et al., 1995) and epidermolysis bullosa (Christiano & Uitto, 1996).

Lundgren et al., (1985), found that after 2 weeks of culture, myocytes seeded on dishes coated with laminin formed a denser monolayer than those cultured on uncoated

plastic dishes. These also exhibited spontaneous contraction. Carvalho et al., (2003), demonstrated enhanced adherence and osteopontin expression of chicken calvarial osteoblasts cultured on laminin coated polystyrene. They also concluded that of several substrate coatings studied, laminin was the most efficient enhancer of osteoblast attachment. Roessler et al., (2001), found that bonding of MC3T3-E1 cells to titanium dental implants was enhanced by a linear adhesion peptide developed from the laminin sequence. Interestingly, microarray gene expression studies of MC3T3-E1 cells by Beck et al., (2001), identified high levels of expression of laminin receptor 1. These reports suggested that laminin could be a further potential modifier of the osteoblast phenotype.

1.5.8 Cell Strategies

The bio-material composite model for tissue engineering requires a source of cells with which to seed material scaffolds. Just as the ideal material should possess certain characteristics, so an ideal profile for any cell type utilised, exists. They should be non-immunogenic, easily and rapidly expandable in culture and accessible with minimal donor morbidity and risk (Warren et al., 2004). Cells used may be drawn from a range of sources, including primary tissues or cell lines such as the murine, calvaria derived MC3T3-E1 osteoblast like cell line. This line which will be discussed more fully in chapter 4 has been well characterised in terms of its temporal expression of osteoblast related proteins and has been widely used in studies of osteogenisis.

Primary tissues may be autologous (from the same individual), syngenic (from a genetically identical individual), allogenic (from a different individual of the same species) or xenogenic (from a different species). However, the use of xenogenic or allogenic cells is limited by the need for host immunosupression, although techniques to render cells immunologically “invisible” may make them a clinical reality for the future.

An area of research which is rapidly developing, involves the potential use of embryonal stem cells, although ethical issues and ongoing medico-political debate regarding the further development and implementation of this source, renders them currently inaccessible (Zoloth, 2002; Walters, 2004). Therefore, cells derived from bone marrow aspirates are the most likely therapeutic candidates for host derived cells. They are capable of developing cell lineages producing adipose, muscular and cartilaginous tissues as well as bone (Prockop, 1997). Shen et al., (2002), has used systemically administered bone marrow derived cells to enhance healing of long bone fractures in mice. The remarkable potential to use cells from liposuction of fat, a readily available source, has

also been reported when Zuk et al., (2001), demonstrated the multi-lineage potential of human derived adipose cells.

A further potential source of cells in patients would be those derived from the site of surgery. This would require subsequent enzymatic digestion and culture expansion of osteogenic cells in much the same fashion that cells are currently derived for research. This approach however, would only be feasible on ethical grounds, for individuals having planned multi-staged procedures.

1.6 Hypothesis and study strategy

The need for reconstruction of bony defects following surgery in children with syndromic craniosynostosis has been established. Very little is known about the behaviour, interactions and ability of osteoprogenitors carrying FGFR mutations to produce viable bioscaffolds, which would appropriately regenerate bony defects.

The working hypothesis for the study was that:

Osteoprogenitors carrying mutations for craniosynostosis could be grown on bioresorbable scaffolds to regenerate bony calvarial defects.

The ultimate long term goal of regenerating critical size cranial bone defects, of which this study would be an initial part, would require several steps.

In the first instance, the study involved working exclusively with the MC3T3-E1 cell line. An important first step was to further characterise the osteogenic phenotype of this line, transfected with the FGFR2-C278F mutation for craniosynostosis and wild type human FGFR2. This would help clarify whether this model was a suitable one in which to progress the further work required toward the long term clinical aim.

To accomplish this, the examination of mRNA expression of matrix proteins produced by the osteoblast phenotype was undertaken using RT-PCR. Subsequently genes which may have been significantly regulated by the mutation, given the observed behaviour and morphological appearance of the mutated line were studied. An indirect immunohistochemistry technique was utilised to assess proliferation of the cell lines.

Having observed and further characterised the effect that the mutation was having on the phenotype, these findings directed the attempt at pharmacological manipulation of the mutated cells, in order to revert or rescue them towards the wild-type (Chapter5). It was then appropriate to study the growth of these cells on a bioabsorbable scaffold, whilst exploring potential substrates to enhance osteogenisis. (Chapter 6)

A novel, composite material incorporating an expanded formulation of polylactide-co-polyglycolide combined with a bioactive glass had been pledged at the beginning of the project. Unfortunately as the study reached the stage where it was feasible to proceed to investigate cellular behaviour on the scaffold, it was not made available. Therefore, following discussion with study supervisors, it was elected to substitute this novel material with PLGA plates (80:20, PLA:PGA) (Bionx, Tempere, Finland), which were already being used for craniofacial reconstruction/fixation in our institution and which were therefore readily available.

Towards the end of the study, the first formulation of this novel material became available and only initial pilot studies of cellular growth on this material were possible.

The research questions which were to be answered in an attempt to fulfil those first steps were therefore:

- Did the stably transfected FGFR2-C278F cell line proliferate then differentiate to express markers appropriate to an osteoblastic phenotype and subsequently mineralise.
- Could this cell line attach, proliferate and mineralise on a bioabsorbable membrane in an attempt to assess its usefulness as a model for future study of bio-composite bone engineering in craniosynostosis.
- Could the attachment, growth and differentiation of these mutated osteoprogenitor cells on bioresorbable scaffolds be optimised for osteogenisis.

Chapter 2: Materials

The manufacturers of all materials used are detailed below. The region and country of the manufacturer is given with the product description. Where the same manufacturer has supplied multiple items, the region and country will be shown once. Subsequent references to the same manufacturer will show only the country of manufacture.

2.1: Murine cell lines

MC3T3-E1 pre-osteoblasts and MC3T3-E1 cell lines stably expressing either Human Fibroblast Growth Factor Receptor 2 (referred to subsequently as “wild-type”- WT) and a mutated form of the receptor (referred to subsequently as “C278F”) coding for the C278F missense mutation (Oldridge et al., 1995), were used. Transfection had been carried out in pcDNA3 plasmid (Invitrogen, Carslbad, California) by electrocorporation and selected using 200µg/ml of Zeomycin to establish stable cell lines (Santos-Ruiz et al., 2007). All lines were stored in liquid nitrogen and thawed as required.

2.2: Tissue culture media and equipment

Alpha modification of Eagle’s minimal essential medium with ribosides and deoxyribonucleosides (α-MEM) and Dulbecco’s calcium, magnesium and sodium bicarbonate-free phosphate buffered saline (DPBS), L-Glutamine, Penicillin/streptomycin solution (100,000 units per ml), and foetal calf serum (FCS) were all supplied by Gibco BRL (Paisley, UK). Bovine or calf serum (FBS and FCS, respectively) are the most widely used growth supplements for cell culture, primarily because of their high levels of growth stimulatory factors and low levels of growth inhibitory factors. Maintaining successful and consistent cell fermentations can be difficult, as FBS and FCS are complex natural products and may vary from lot to lot even from a single manufacturer. Moreover, the quality and concentration of both bulk and specific proteins can affect cell growth. Therefore, batches of FCS were tested at the start of the project and a single batch ordered and used throughout, in all cultures.

Dexamethasone, b-glycerophosphate and ascorbic acid were purchased from Sigma-Aldrich (Gillingham, UK).

Trypsin –EDTA for cellular dissociation was purchased from Sigma-Aldrich (UK).

Tissue culture grade Petri-dishes, multiple well plates and other plastics were supplied by Philip Harris (Stoke, Staffordshire, UK), Nunc Inc (Roskilde, Denmark) and Helena Bioscience (Sunderland, UK).

2.3: Substrate coating agents

Laminin (L2020), and fibronectin (F2006) for coating of surgical plates and culture dishes were obtained from Sigma-Aldrich (UK).

2.4: Growth factors

Human recombinant FGF18 (F7301) was purchased from Sigma-Aldrich (UK)

2.5: Bioabsorbable membranes

Surgical grade polyglactide plates used for craniofacial fixation were purchased from Bionx Implants Ltd (Tempere, Finland). Two formulations of Bioglass/$PLGA_{80}$ composite membrane were a gift from Bionx Implants Ltd.

2.6: Primary antibody

Anti-phospho-Histone H3 (mitosis marker) rabbit polyclonal IgG was obtained from Upstate Biotechnology (Buckingham, UK). In all experiments, the antibody was used at a dilution of 1 in 400. The immunogen used for the antibody production was a KLH-conjugated peptide corresponding to amino acids 7-20 of the human histone H3, which recognises the phosphorylated form of histone H3 in vertebrate species

2.7: Secondary antibody

Goat anti-rabbit immunoglobulin was obtained from DAKO Ltd (UK). In all experiments, the antibody was used at a dilution of 1 in 100.

2.8: Sera as blocking agent

Goat serum was obtained from DAKO Ltd (UK).

2.9: Other immunohistochemical detection reagents

StreptavadinABComplex/HRP (ABC duet kit), used for the labeling and signal amplification of secondary antibodies used in H3 staining was purchased from DAKO Ltd (UK). Diaminobenzidine tetrahydrochlorodihydrate (DAB) tablets, Triton X100 and paraformaldehyde(PFA) were obtained from Sigma-Aldrich (UK). Alkaline phosphatase activity was detected by NBT/BCIP purchased from Roche, (Basel, Switzerland). The combination of BCIP (5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt) and NBT (Nitro-Blue Tetrazolium Chloride) yields an intense, insoluble black-purple precipitate when BCIP is hydrolyzed by alkaline phosphatase to form an intermediate that undergoes dimerisation to produce an indigo dye.

2.10: Microscopy and image capture

Cultured cells were observed under a Zeiss SV11 inverted microscope, using objective lenses of 2.5x, 10x and 32x and photographed using a Yashika 108 multi program 35mm single lens reflex camera and Kodak TMax 160 colour reversal film or Fujichrome T64 colour reversal film. Brightfield microscopy was performed on a Zeiss Axiovert135 microscope (Carl Zeiss, Germany) using objective lenses of 2.5x, 10x, and 20x . Digital images were captured electronically using a Hamamatsu digital camera (C4742-95, Hamamatsu Photonics KK, Japan), and opened into Openlab software version 3.05 (Improvision, Coventry, UK). Images were saved in formats compatible with Adobe Photoshop version 6.0 (Adobe Systems Europe, Edinburgh, UK). Images taken on colour reversal film were scanned digitally using an Epson Film Scan 200 slide scanner and the associated Twain 32 software (Epson, Hemel Hempstead, UK). Images were processed using Adobe Photoshop version 6.0 where necessary (Adobe Systems Europe, UK) and saved to a Macintosh G3 computer.

2.11: Cell Tracker probes

5-Chloromethylfluorescein diacetate (CMFDA) vital cell-tracking dye was obtained from Molecular Probes (Invitrogen, USA).

2.12: Mineral detection

Alizarin red, Silver nitrate and sodium thiosulphate were purchased from Sigma-Aldrich (UK).

2.13: General laboratory reagents

The general reagents used for experimental procedures were Analar grade supplied by BDH Ltd (UK) unless otherwise stated. Sigma-Aldrich (UK) supplied diethylpyrocarbonate (DEPC), ethidium bromide, orange G, paraformaldehyde (PFA), citric acid and hydrogen peroxide. Phosphate buffered saline (PBS) tablets were purchased from Oxoid (Hampshire, UK).

2.14: Gel electrophoresis and molecular size markers

Agarose powder was supplied by Gibco BRL (UK). Agarose gels were run in the Horizon horizontal gel electrophoresis system (Gibco BRL, UK). 1Kb ladders for RNA analyses were either from Gibco BRL (UK), or Bioline (London, UK). Trisma base, glacial acetic acid and EDTA (used to make Tris acetate EDTA electrophoresis buffer-TAE) were purchased from Sigma Aldrich (UK). Gel images were captured using AlphaImager™1200 computer software (Alpha Innotech Corporation, San Leonardo, Ca., USA).

2.15: Reagents and equipment for RT PCR

Reverse transcriptase, random hexamer primers, deoxynucleoside triphosphates (dNTPs), RNA inhibitor, magnesium chloride and 10x buffer set, and Taq polymerase were purchased from Promega (Southampton, UK). Thermostable PCR tubes were purchased from Elkay Laboratory Supplies Ltd. (Hampshire, UK). PTC-1000 thermal cycler machine was from MJ Research Inc. (Boston MA, USA).

2.16: Restriction Enzyme

Following a restriction site search using the Promega search resource at: www.promega.com/guides/re_guide/research.asp?search=seq, the restriction enzyme *BbsI* was purchased from New England Biolabs (Hertfordshire, UK) to confirm the presence of the C278F mutation.

2.17: Oligonucleotides

Oligonucleotides were custom synthesised by Genosys (Cambridge, UK). The oligonucleotides presented in **Table 2.1** were each designed from published records of cDNA using the Pubmed nucleotide enquiry facility on:
www.ncbi.nlm.nih.gov/genome/seq/BlastGen/BlastGen.cgi?taxid=10090
The appropriate sequence was then used to design the oligonucleotide forward and reverse primers with the aid of PRIMER3 software, based centrally at Massachusetts Institute of Technology on: http://fokker.wi.mit.edu/primer3/input-030.htm

2.18: Centrifuges

Samples were centrifuged in a Heraeus Biofuge 13R (Heraeus instruments, UK) or a Sigma 113 microfuge (Sigma, Osterode, Germany).

AlkP	AF285233	**403bp**	**56°C**
Forward	*192-211*	5’ ATGGGCGTCTCCACAGTAAC 3’	
Reverse	*595-576*	5’ TTATCCGAGTACCAGTCCCG 3’	
Col1	U08020	**283bp**	**56°C**
Forward	*293-212*	5’ GCTTCAGTGGTTTGGATGGT 3’	
Reverse	*576-557*	5’ AGAGCCTCTAGCTCCTTGGG 3’	
Cx43	M61896	**468bp**	**56°C**
Forward	*143-162*	5’ GGGTGATGAACAGTCTGCCT 3’	
Reverse	*602-621*	5’ GAGAGGAAGCAGTCCACCTG 3’	
FGF18	NM8005	**267bp**	**56°C**
Forward	*190-209*	5’ CCTGCACTTGCCTGTGTTTA 3’	
Reverse	*457-438*	5’ AGCCCACATACCAACCAGAG 3’	
GapDH	L26492	**396 bp**	**55°C**
Forward	*151-170*	5’ ACTCCACTCACGGCAAATTC 3’	
Reverse	*547-528*	5’ GTCATGAGCCCTTCCACAAT 3’	
hFGFR2	Z 71929	**630bp**	**56°C**
Forward	*413-432*	5’ ATGGTGCGGAAGATTTTGTC 3’	
Reverse	*1042-1023*	5’ TAGAATTACCCGCCAAGCAC 3’	
NCad	M31131	**306bp**	**56°C**
Forward	*634-653*	5’ CGGTTTCACTTGAGAGCACA 3’	
Reverse	*939-920*	5’ CACAGTGATGATGTCCCCAG 3’	
OC	L24431	**214bp**	**60°C**
Forward	*51-70*	5’ TGAGGACCCTCTCTCTGCTC 3’	
Reverse	*264-245*	5’ GCGTCTGTAGGCGGTCTTTA 3’	

Table 2.1 : Oligonucleotide sequences of primers used for reverse transcriptase polymerase chain reaction (RT-PCR)

The accession number is underlined. Bold characters indicate the expected product size in base pairs. Italics indicate the position of the primer sequence relative to the position of the corresponding base pairs in the cDNA sequence. Primer annealing temperatures used are shown to the right of the base pair number. All primers are murine cDNA sequence derived other than hFGF

Chapter 3: Methods

3.1: Cell Culture

3.1.1: Sterile technique and culturing

All tissue culture plates, bioabsorbable membranes, dishes, pipettes, containers and solutions used were sterile. All aliquotting and preparation of plates was performed under a laminar flow tissue culture hood which had been switched on at least 30 minutes prior to use, and cleaned with 70% industrial methylated spirit (IMS)/dH_20. Clean nitrile gloves were used during all procedures, and these were regularly sprayed with 70% IMS and changed periodically. Instruments were flamed in 100% IMS, under the laminar flow hood, and allowed to cool before use. Manipulation and washing of Bionx plates was carried out under sterile conditions throughout.

All media and additives prepared and aliquotted into Falcon tubes and dishes were kept under the laminar flow hood prior to seeding, splitting or feeding of cultures. Cells were harvested for passage or RNA extraction as follows: Medium was removal from flasks, adherent cells were washed twice with warmed phosphate buffered saline (PBS) and released with 0.5% trypsin-EDTA. Cultures were maintained in a humidified incubator in a mixture of 95% air and 5%CO_2 at 37^{o}C. Unless stated otherwise, cells grown for 30 day osteodifferentiation cultures were grown in T-25 flasks, seeded at 12,000 cells/cm^2. Cells grown for pre-confluent studies were seeded at 5000 cells/cm^2 in T-75 flasks to maximise biomass for RNA extraction. Unless otherwise stated, all cells for preconfluent studies were cultured in standard medium to maximise proliferation (αMEM, 10%FBS, 2mM L-glutamine, 100U/ml penicillin, 50µg/ml streptomycin) Cultures for differentiation studies were supplemented with 50µg/ml ascorbate and 10mM ß-glycerol-phosphate when cells became fully confluent.

3.1.2: Freezing and Thawing

Freezing cells involved harvesting them as described above, suspending them at a concentration of 10^6/ml in freezing medium containing 10% dimethylsulphoxide (DMSO) and 20% FBS, aliquoting 1 ml of cell suspension into cryotubes, slow freezing in a -20^{o}C freezer, transferring and storing the cryotubes overnight at -80 °C and transferring them to a liquid nitrogen container the next day.

To thaw frozen cells, vials were removed from liquid nitrogen storage and immediately placed in a 37°C water bath. The cell suspension was transferred by pipette to a 15-ml falcon tube and 5 ml of warm medium was added slowly to the tube and mixed by gentle repeat aspiration. Aliquots of cell suspension were transferred to flasks containing additional medium and cultured under standard condition. Complete medium changes were performed after 12 hours of culture and subsequently every 48 hours.

3.1.3: Cell number and viability

Medium from plates, wells or flasks was removed; adherent cells were washed twice with (PBS) and released with 0.5% trypsin-EDTA solution. Cell number and viability were determined by haemocytometer count and trypan blue exclusion according to accepted conventions for cell counting in grids, namely, viable cells crossing the upper and right hand grid lines are counted and those on the lower and left hand lines are excluded (**Fig. 3.1**)

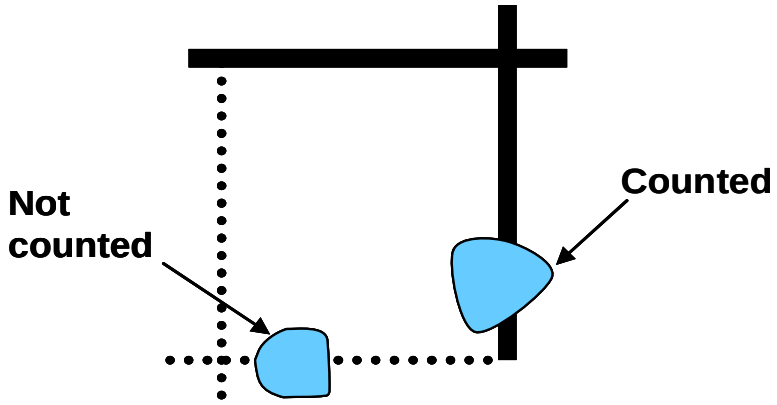


Figure 3.1: Haemocytometer cell counting convention

3.2: Vital cell tracking

Cells difficult to visualise under bright field microscopy were treated with a vital, intracellular, fluorescent dye to allow observation under ultra-violet light. Live cells were labelled with CellTracker Green (CMFDA), according to the manufacturer's instructions. Cells were incubated at 37^{o}C for 45min in labelling solution (1 µM CMFDA in prewarmed, serum-free medium), followed by a 45 minutes incubation in normal medium (without CellTracker). Cells were gently washed with PBS. Labelled cells were put back into the incubator in normal medium for observations at 520 nm wavelength.

3.3: Cellular proliferation assay

One of the first steps in cell division is the replication of DNA during S-phase of the cell cycle. Cellular proliferation in cultured cells can be assessed using various techniques. Direct counting of mitotic cells in haematoxylin and eosin (H&E) stained sections can be a difficult and time-consuming process. It is especially difficult to distinguish between cells in prophase and cells undergoing pycnosis or apoptosis in H&E-stained samples. High magnifications have to be used to distinguish the cycle phases and large numbers of cells must be counted to obtain statistical validity. This has led to the development of specific techniques and markers to immunologically identify cell division.

The anti-phosphorylated-H3 antibody is one such method, which recognises histone H3 after it becomes phosphorylated when the chromosomes condense during cell division. This technique has been shown to be a sensitive and reliable method for assessing cells undergoing mitosis (Brenner et al., 2003).

This assay utilises the well established technique of immunohistochemistry, which relies on the highly specific binding properties of mammalian antibodies to recognise protein epitopes in fixed cell culture or tissue sections (Polack & van Noorden, 2003).

The indirect technique for detection of the targeted epitope was used (**Fig. 3.2**). This method requires a primary and a secondary antibody as well as a visualisation marker to allow counting of the proliferating cells.The primary antibody is usually an IgG raised against the epitope of choice, in a mammalian species different from the sample species. The secondary antibody is obtained from a different species to that of the primary antibody and conjugated with peroxidase to allow signal detection (**Fig 3.2A**) in combination with an avidin-biotin amplification complex and visualisation compound such as diaminobenzidine-tetrahydrochloride (DAB) (**Fig. 3.2B**). Further important steps to block endogenous peroxidase (**Fig. 3.2C**), charged proteins and non-specific antigens (**Fig. 3.2D-E**) are carried out to reduce false positive detection and minimise background staining.

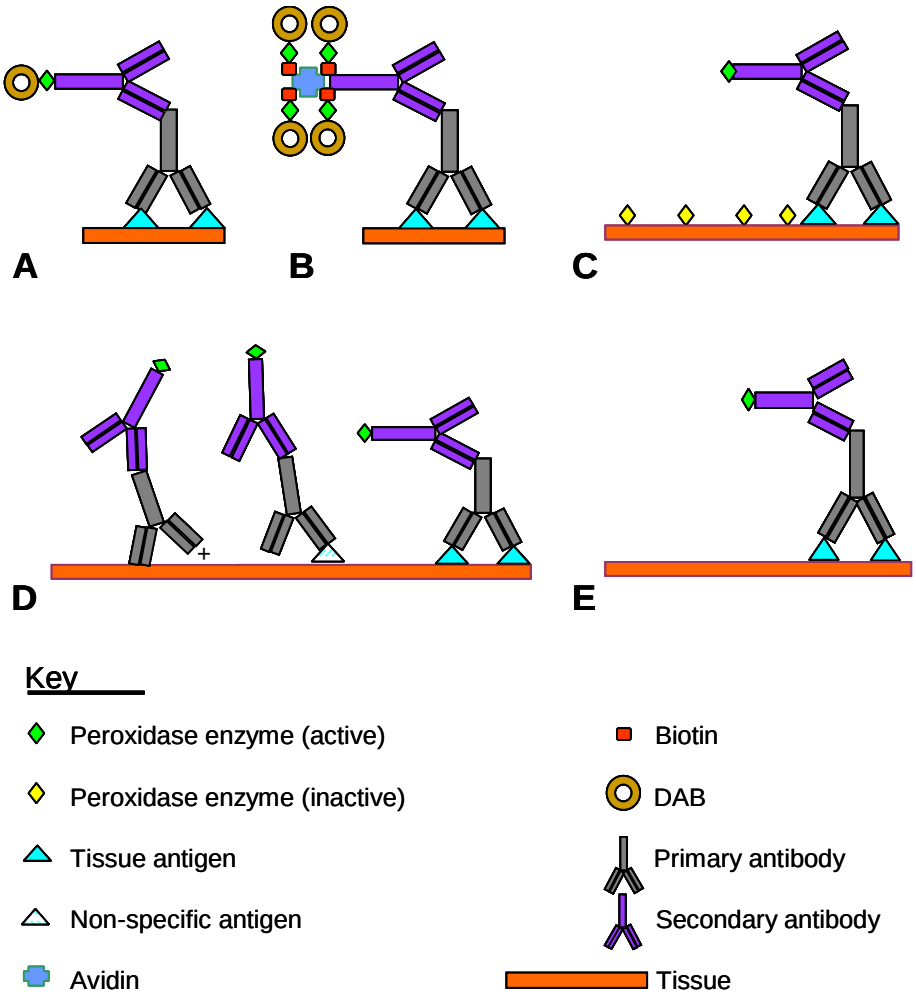


Figure 3.2: Indirect method of immunohistochemical antigen detection

A: The primary antibody binds the targeted epitope which in turn is bound by peroxidase conjugated secondary antibody. The peroxidase activity is detected by colour development by incubation with a substrate such as DAB. **B**: Amplification steps using avidin-biotin complex can be included to strengthen the signal prior to DAB exposure. **C**: Hydrogen peroxide is used to block endogenous peroxidase activity within the tissues. **D**: Charged proteins and non-specific epitopes may react with primary antibody, leading to background staining. **E**: Treatment with protein-rich serum blocks these non-specific epitopes to provide a “clean” signal.

3.3.1: Cellular Fixation

Cell cultures were washed once with PBS and fixed by cold 4% paraformaldehyde (PFA) in PBS for 30 minutes at room temperature. The PFA was removed and the cells washed with cold PBS for 10 minutes, 3 times by placing on a rocker-table agitator.

3.3.2: Blocking

Endogenous peroxidase activity was then quenched by incubation of the cells with 3% hydrogen peroxide (H_2O_2) in 10% methanol in PBS at room temperature for 20 minutes. The H_2O_2 was removed and the cells washed for 5 minutes, 3 times in PBS. Non-specific proteins and charged compounds were then blocked by saturating the cells in a 10% solution of goat serum in PBS for 45 minutes at room temperature. The serum was removed and the cells washed 3 times in PBS at room temperature.

3.3.3 Application of primary antibody

The cells were coated and incubated with a layer of anti-phosphorylated-H3 antibody at a dilution of 1:400 in PBS overnight at 4^oC. Negative controls were treated similarly without the addition of primary antibody. The following day, samples were thoroughly washed and agitated on a rocker-table 3 times with 0.5% Triton in PBS, to ensure only specifically bound primary antibody remained, prior to application of the secondary antibody. The cells were washed a further 3 times in PBS to remove traces of Triton.

3.3.4: Application of secondary antibody

The cells were then incubated for 45 minutes at room temperature with goat anti-rabbit IgG diluted to 1:100 in PBS. They were then washed 3 times with PBS before detection of the secondary antibody.

3.3.5: Antibody detection

Avidin-biotin-peroxidase complex was prepared from an ABC kit according to the manufacturers instructions, diluted with PBS and allowed to stand for 30 minutes. It was then added to the samples for 30 minutes before removal and washing with PBS. This amplification kit confers a fourfold increase in the levels of peroxidase available for development. Visualisation of the peroxidase tag was then detected by colorimetric changes due to precipitation of its substrate diaminobenzidine-tetrahydrochloridehydrate (DAB) which was made up into 5ml solutions with MilliQ water according to manufacturer's

instructions and filtered through a 0.4 µm pore filter. Half a mililiter of DAB was added to each well and allowed to develop for a fixed period for all wells as ascertained by pre-testing. The DAB solution was removed and wells washed for 5 minutes in PBS before image capture.

3.3.6: Proliferation count

Once the plates had been processed, triplicates of four random areas per well were captured digitally, downloaded and stored as TIFF image files onto a Mac G3. The total number of cells per field and the number of H3 positive nuclei for that field were counted using the Openlab software point counting option. Briefly, the computer mouse was used to 'click' on each cell. Each 'click' labelled the cell with a mark and a number, so that the user could see which cell, and how many, had been counted for any particular field of view. To optimise objectivity of H3 counting, the gain/contrast control on the software was then adjusted to exclude visualisation of cells not positive for H3 and the counting process was repeated (**Fig. 4.6A**). This system was adopted to reduce counting error. The counts were tabulated using Microsoft Excel software, where the H3 positive cells were expressed as a percentage of the total cell count, using the following equation:

$$\text{Mitotic Index} = \frac{\text{number of H3 positive nuclei}}{\text{total number of cells in field}}$$

The data was separated into controls and FGF18 treated cells and transferred into SPSS (10.0) for statistical analyisis. Proliferation rates were analysed for statistical significance using analysis of variance between groups (ANOVA) and post hoc tests were carried out using Bonferroni's method.

3.4: Growth factor treatment

Human recombinant fibroblast growth factor (FGF) 18 was obtained from Sigma-Aldrich (U.K.) and used for the treatment of cultured cell lines. Concentrations of FGF 18 between 10^{-10}M and 10^{-8}M were first prepared to ascertain a treatment range for experimentation. The level of foetal calf serum (FCS) required to maintain viability of the cells during experimentation was determined at 24 and 72 hours post treatment, using FGF18 at a concentration of 10^{-9}M.

For experiments, triplicate samples of cell lines were seeded at 12,000 cell/cm^2 in normal culture medium. This was replaced after 12 hours following washing in PBS at 37^{o}C, by experimental medium containing 2% FCS and FGF18 at differing concentrations.

Proliferation assays using anti-phosphorylated-H3 were carried out as described in section 3.3 and a mitotic index calculated for each treatment condition

3.5: Bioabsorbable membranes

Bioabsorbable fixation plates or composite membranes provided by Bionx (Finland) were removed from sterile packaging and manipulated under the tissue culture hood at all times. Materials were divided for experimentation into equal portions, marked and cut with a scalpel blade (**Figure 3.3**). The materials were placed in tissue culture wells and the wells seeded with cells at the experimental concentration. For experiments lasting several days, cells were allowed to attach for 12 hours before gently washing the materials with PBS at 37^{o}C to remove unattached cells and placed in a fresh well and medium to allow only cells originally attached to the material to be included. Medium was changed every 48 hours. For measurement of seeding efficiency with membrane coatings, the material was removed from its original well, gently washed in PBS at 37^{o}C and cells detached with warmed 0.5% trypsin-EDTA. Cells were counted as described in section 3.1.3. Staining for markers of mineralisation was carried out as described in section 3.7.

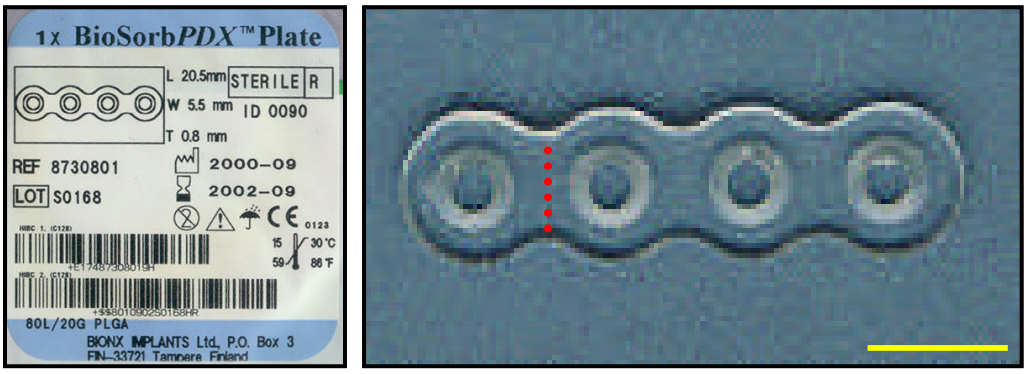


Figure 3.3 : Commercially available resorbable bone fixation plates

A. BioSorbPDX, co-polymer, Polyglycolic(20%)/Polylactate(80%) plates supplied as sterile packed implant. 800µm thick with 1.5mm screw holes.

B. Supplied plates were divided by sharp, sterile scalpel to provide single hole fragments of 5mm diameter. Scale bar = 5mm

3.6: Membrane coatings

Pre-treatment of culture dishes and bioabsorbable membranes with fibronectin or laminin was performed by adding 0.5ml of 0.01% solutions of laminin or fibronectin to tissue culture wells to allow total surface wetting, followed by removal of the excess. Dishes were then allowed to air dry for 1 hour under the tissue culture hood, prior to seeding of cells. PLGA bioabsorbable plates were coated by immersion into a solution of either laminin or fibronectin at the above concentration, allowed to drip excess for 2 minutes then transferred to a culture plate to air dry before transferring to the centre of a fresh culture well prior to the seeding of cells. Cells were released for collection or counting as described in section 3.1.3.

3.7: Staining for mineralisation

3.7.1: Von Kossa

This method relies upon the principle that silver ions can be displaced from solution by carbonate or phosphate ions due to their respective positions in the electrochemical series. The argentaffin reaction is photochemical in nature and the activation energy is supplied from strong visible light. Since the demonstrable forms of tissue carbonate or phosphate ions are invariably associated with calcium ions, this method can be considered as demonstrating sites of tissue calcium deposition. Cultures were washed 3 times in PBS for 2 minutes on a rocker-table. Cells were then fixed with 4% PFA in PBS for 30 minutes and washed 3 times with 10mM Tris-HCl, pH 7.2. Fixed cells were incubated with 5% silver nitrate for 1 hour under a halogen lamp, washed 3 times in PBS and then treated with 5% sodium thiosulphate. Cells were imaged using bright field microscopy and images captured on colour reversal film.

3.7.2: Alizarin Red S

Following removal of medium, plates were gently washed with PBS 3 times and fixed with 4% PFA for 30 minutes, The PFA was removed and the plates washed again 3 times with PBS. Alizarin Red S stain was added at a concentration of 1% at pH 4.2 for 10 minutes at room temperature, then washed 3 times with distilled water and once with PBS before visualisation with bright field microscopy and image capture with colour reversal film.

3.7.3: Alkaline phosphatase

Alkaline phosphatase activity was detected by NBT/BCIP staining. Plates were gently washed once in PBS and then fixed with 4% PFA in PBS. They were then washed 3 times for 5 minutes in PBS at room temperature on a rocker-table and incubated for 10–30 minutes at room temperature in 0.0375 µg/ml NBT + 0.0188 µg/ml BCIP dissolved in 100 mM Tris–HCl buffer, pH 9.5, containing 5 mM MgCl2. All plates were developed for the same period of time. The reaction was stopped by washing 3 times in buffer followed by 3 times in distilled water. Plates were then imaged using bright field microscopy and captured on colour reversal film

3.8: RNA analysis

All solutions for RNA work were made using diethylpyrocarbonate (DEPC) treated water. DEPC is a potent inhibitor of ribonucleases (RNAse) which rapidly degrades RNA molecules. 1ml DEPC was added to 1L MilliQ water. This was incubated at 37 °C overnight to kill the RNAse. The DEPC in the treated water was finally degraded to ethanol and carbon dioxide by autoclaving. All reagents used for RNA extraction were kept separate from other laboratory agents to reduce the risk of RNAse contamination. RNAse-free equipment was used throughout the procedure.

Total RNA was extracted from cultured cells using TRI-REAGENT™. TRI-REAGENT™ promoted the formation of RNA complexes with guanidinium and water molecules, and inhibits hydrophilic interaction between the DNA and protein. The RNA was isolated in the aqueous phase, which was subsequently removed and purified.

3.8.1: RNA extraction from cultured murine lines

All tissue culture media was removed from the culture flasks or plates. One mililiter of 0.5% trypsin-EDTA was added to each flask or plate and returned to a humidified tissue culture incubator, at 37 °C in 5% CO_2 for 5 minutes. The samples were then gently agitated to aid release of the intact cells which were then collected after the addition of 5 ml of PBS. This suspension was spun at 1000 G for 5 minutes. The supernatant was discarded, and all traces of liquid removed from the cells using a fine glass pipette. 1 ml of TRI REAGENT™ was added to the cellular pellet, and repeatedly pipetted for several minutes and vortexed to promote complete cell lysis. The sample was either stored at -80^{o}C for later RNA extraction or left for 5 minutes at room temperature, treated with 200 µl chloroform, vortexed and centrifuged. Samples were left to stand at room temperature for

15 minutes, in order to optimise the separation of RNA, DNA and proteins and then centrifuged at 12,000 rpm for 10 minutes at 4°C. The top aqueous layer containing the RNA was removed carefully by pipette and put into a separate Eppendorf tube. Five hundred microlitres of isopropanol was added to this in order to precipitate the RNA. The extract was centrifuged at 12,000 rpm for 10 minutes at 4 °C. The resultant RNA pellet was visualised, and the supernatant removed. The pellet was washed gently in 1 ml of 75% ethanol in DEPC water and centrifuged at 12,000 rpm for 2 minutes. The supernatant was removed completely, and the last traces of liquid were left to evaporate from the pellet for several minutes before gently re-suspending the RNA in 20-50 μl of DEPC water. The concentration of RNA was then evaluated by measuring the absorbance at 260 nm wavelength using a Genequant spectrophotometer. The quality of RNA extraction was determined periodically throughout the study by running samples on agarose gel electrophoresis. Intact total RNA run on a denaturing gel will have clear 28S and 18S ribosomal RNA bands (**Figure 3.4 A**) whereas degraded RNA demonstrates smeared, drawn-out bands. While crisp 28S and 18S RNA bands are indicative of intact RNA, visual assessment of the 28S:18S rRNA ratio on agarose gels is somewhat subjective because appearance of RNA bands is affected by electrophoresis conditions, amount of RNA loaded, and saturation of ethidium bromide fluorescence. Hence, the RNA quality was further determined periodically by a colleague testing samples in an Agilent Technologies 2100 Bioanalyzer (**Figure 3.4 B**). This uses a combination of microfluidics, capillary electrophoresis, and fluorescence by the addition of an RNA specific dye, to evaluate both RNA concentration and integrity.

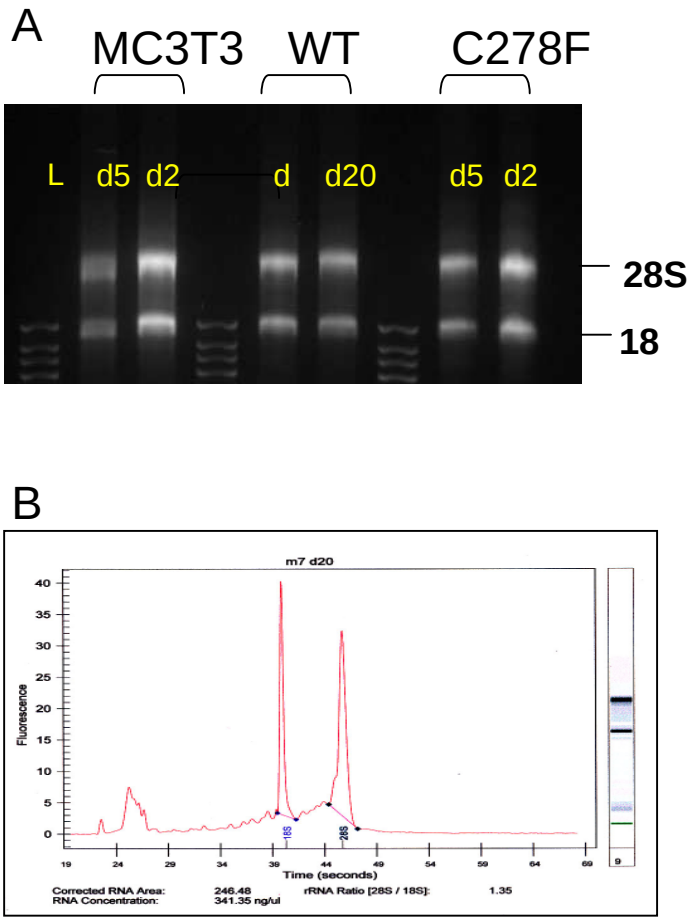


Figure 3.4 Determination of RNA quality

A: Extracted RNA was run on a denaturing gel. Characteristic 18S and 28S bands of ribosomal RNA are clearly demonstrated.

B: 18S and 28S peaks are prominent and RNA concentration is determined by spectrofluorescence.

3.8.2: Reverse transcription polymerase chain reaction

The reverse transcription polymerase chain reaction (RT-PCR) is a powerful molecular technique that is widely used to assess gene transcription in whole embryos, tissues, or isolated cells. The technique allowed for highly specific and sensitive amplification of specific mRNA transcripts. RT-PCR was very useful in the detection of low amounts of mRNA, that would not be detected using other methods such as Northern blots. The RT-PCR method involved an initial reverse transcription (RT) step in which all mRNA transcripts were reverse-transcribed by a retroviral enzyme using specific primers, short random hexamers or oligo-dTs as primer templates (**Fig.3.5**). This process lead to the formation of a set of double helices, each made up of the original mRNA template, and its complementary strand of newly transcribed cDNA. Subsequently, the cDNA of interest was separated from the mRNA and amplified exponentially using gene-specific primers and a thermo-stable DNA polymerase enzyme (**Fig.3.6**).

3.8.2.1: Reverse transcription

Reverse transcription (RT) involved the synthesis of complementary DNA (cDNA) using single stranded RNA as a template, as illustrated in Figure 3.4. The Maloney murine leukaemia virus (M-MLV) reverse transcriptase was used for all RT reactions. Using RNAse-free equipment, 1 μg of RNA, made up to 10 μl with DEPC water, was added to 1 μl of 100 pmol random hexamer primers (pN6) in a 0.5 ml microfuge tube. The tube was placed into a PCR thermal cycler and heated to 70 °C for 10 minutes. This step removed secondary mRNA structure. The reaction mix was cooled rapidly on ice. 9 μl of a mix containing 4 μl of 5x first strand buffer, 2 μl of 100 mM dithiothreitol (DTT), 1 μl of 10 mM deoxynucleoside triphosphates (dNTPs), 1 μl of RNAse inhibitor and 1 μl M-MLV RT (20 U) was added. This was incubated at 42°C for an hour then heated to 95 °C for 10 minutes to destroy the RT enzyme. The RT products were stored at ⁻20 °C until use.

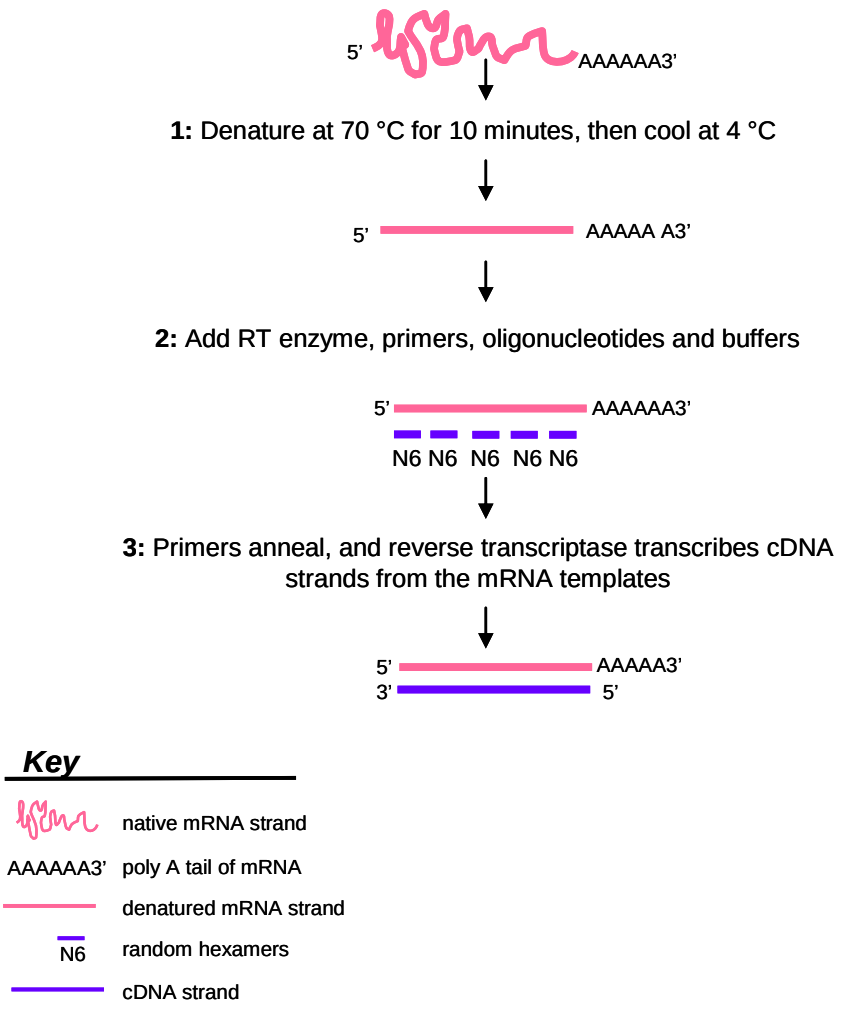


Figure 3.5: The reverse transcriptase reaction

1: Linearisation of extracted mRNA by denaturation. **2**: Addition of reverse transcriptase, random hexamer primers, oligonucleotides and buffer to the reaction mixture. The primers become annealed to their complementary base pairs on the mRNA transcripts. **3**: Transcription of complementary strands of cDNA for each mRNA molecule present in the extract.

(Adapted from Molecular Biology of the Cell, Alberts et al., 2002)

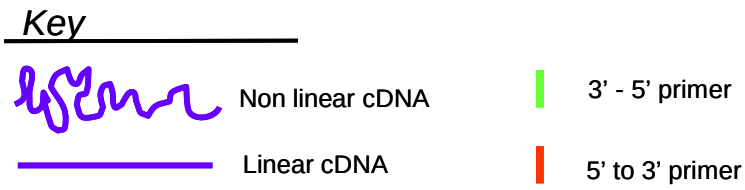
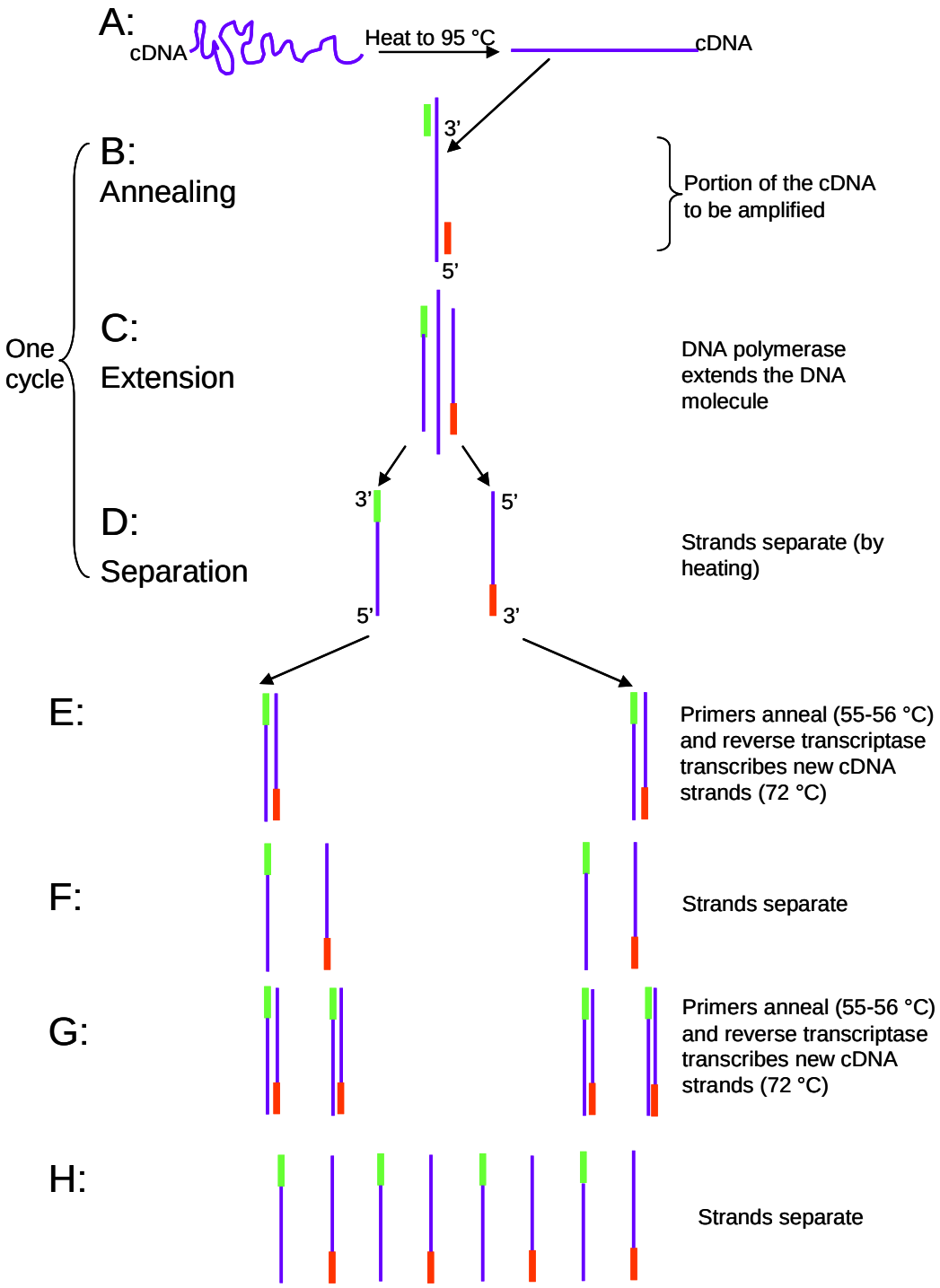


Figure 3.6: The polymerase chain reaction.

A: The cDNA templates were heated to denature and separate the strands. **B:** Upon cooling the reaction mix, primers corresponding to the cDNA of the gene being studied annealed to the cDNA template. **C:** DNA polymerase then transcribed a complementary strand of DNA. **D:** Re-heating the mixture separated the strands. Upon cooling, more primers annealed to the newly transcribed strands, and the process of DNA replication began again (**E**). The process was repeated many times (**F,G,H**) ranging from 24 - 36 cycles resulting in amplification of cDNA strands transcribed. The process was finished with a ten minute polishing step. Samples were stored at 4 °C prior to gel electrophoresis. Adapted from Molecular Biology of the Cell, Alberts et al., (2002).


A:
cDNA
Heat to 95 °C
cDNA
B:
Annealing
3'
5'
Portion of the cDNA to be amplified
One cycle
C:
Extension
DNA polymerase extends the DNA molecule
D:
Separation
3'
5'
5'
3'
Strands separate (by heating)
E:
Primers anneal (55-56 °C) and reverse transcriptase transcribes new cDNA strands (72 °C)
F:
Strands separate
G:
Primers anneal (55-56 °C) and reverse transcriptase transcribes new cDNA strands (72 °C)
H:
Strands separate

3.8.2.2: Polymerase chain reaction

The Polymerase chain reaction (PCR) was used to amplify and detect specific cDNA molecules from the many that had been reverse-transcribed from the mRNA template during reverse transcription. This specificity was attained by the binding of primer pairs to the cDNA template of interest (**Fig.3.6**). The integrity of RNA used in each RT reaction was verified by amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a house keeping gene believed to be expressed in all cells at constant levels.

3.8.3: Primer design

Primers were designed from cDNA records published in original papers, or from an online 'Pubmed Nucleotide Enquiry' (**Table 2.1**). Primers consisted of 19-22 bases, and flanked specific regions of the cDNA of interest, usually spanning a region of 350 to 650 bases. cDNA sites were chosen to cross intron/exon boundaries, in order to detect potential contamination by genomic DNA. The expected product sizes for genomic DNA and cDNA would be very different, allowing detection of genomic DNA contamination of the sample. Primers were selected to have approximately 55% guanidine or cytosine residues. Complementarity between primers was avoided to prevent the formation of primer dimers, which could disrupt the efficiency of the polymerase chain reaction. Generally, the primer concentration was 50 pM in a 50 μl reaction.

The primer annealing temperature can affect the stringency of primer/cDNA interaction. The optimal annealing temperature was calculated from the nucleotide composition of the primer using the following formula:

$$\text{Annealing temperature } (°C) = 4\,(G+C) + 2\,(A+T) - 5.$$

The temperature calculated was not always that used for the primers in this study, although it was a convenient starting point for optimising the temperature for each primer. Annealing temperatures were optomised by running a PCR for each set of nucleotides at $1^{o}C$ increments above and below the formulaic derived temperature. Annealing temperatures of primer pairs were kept the same, thus permitting annealing (and therefore transcription) at the same rate for each strand.

Having chosen the optimum annealing temperature for each primer pair, it was important that visualisation of bands under U-V light should be within the linear intensity spectrum for any particular gene being expressed. Therefore for every gene being studied, a linear range diagram was drawn using 5-cycle PCR increments. The mid-point between

non-detection and maximum intensity was chosen as the number of cycles (n) for each individual gene being studied (**Figure 3.6**).

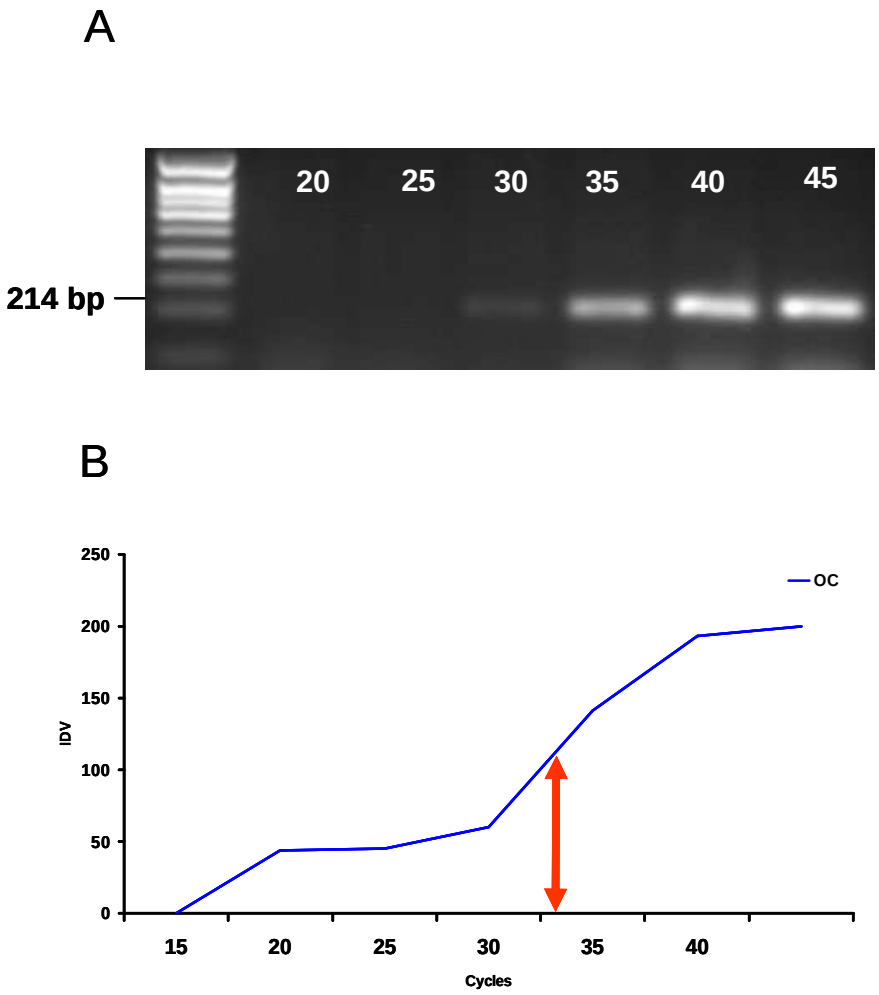


Figure 3.6: Linear range of band intensity for Osteocalcin (OC)

A: PCRs products observed under U-V light at 5 cycle increments

B: Band intensities are measured using Alphaease software and a linear range diagram constructed. The mid-linear point is chosen as the number of PCR cycles for this gene (n = 33 for OC)

A ‘master mix’ of PCR reagents was made up at 4 °C prior to dispensing into separate tubes and the addition of cDNA. This was to ensure that all tubes had exactly the same proportions of PCR reagents. The master mix for a typical 25 μl reaction is given in **Table 3.1**. Four microlitres of cDNA (50 μl reaction) or 2 μl cDNA (25 μl reaction) was then added to each tube. These were placed into a thermal cycler with a heated base and lid for DNA amplification. **Table 3.2** illustrates a typical PCR cycle programme. Samples were kept at 4 °C prior to band separation and detection using agarose gel electrophoresis.

Reagent	**1 X 25 μl reaction**
DEPC water	15.9 μl
$MgCl_2$	1.5 μl
$MgCl_2$-free buffer	2.5 μl
dNTPs 2.5 mM	2.0μl
Forward primer	0.5 μl
Reverse primer	0.5 μl
Taq polymerase	0.1 μl
Total per reaction	**23 μl**

Table 3.1: PCR reagents

The reagents were first made up as a master mix, before addition to individual PCR reaction tubes. This maximised consistency of reagents between reactions. 2 μl cDNA was added in a 25 μl reaction mix and 4 μl cDNA was added per 50 μl reaction mix.

	Temperature	Time	Function
Cycle number (n)	1) 95 °C	5 minutes	(denatures secondary structures)
	2) 95 °C	30 s	(separates cDNA strands)
	3) 55-56 °C	30 s	(primer annealing)
	4) 72 °C	40 s	(Taq transcribes cDNA)
	5) 72 °C	10 minutes	(completion of all cDNA strands)

Table 3.2: Normal thermal cycling programme

The thermal cycler follows steps 1 to 6, cycling 'n' times between steps 2 and 4, before advancing to the polishing step (5)

3.9: Mutation confirmation with restriction enzyme

Restriction enzymes, mostly purified from bacteria, recognise specific DNA sequences and cleave the DNA either within the sequence or at a specific site flanking that sequence. Therefore the number and size of the fragments produced by the digestion depend on the frequency and position of the restriction sites.

The restriction enzyme *BbsI* was used periodically to confirm the presence and absence of the mutation within the C278F and WT cell lines respectively. Following amplification of the hFGFR2 gene, the RT-PCR products were then incubated with *BbsI* at room temperature for 30 minutes. The products were run on agarose gel and imaged as described in section 3.10 and 3.11.

3.10: Agarose gel electrophoresis

Agarose gels for electrophoresis of amplified cDNA were made by heating 1.2 g pure agarose in 100ml tris-acetate-EDTA electrophoresis buffer (TAE) with 0.0025% ethidium bromide (1 μl /ml) for 1.5 minutes at full power in a 700 W microwave oven. The liquid gel was allowed to cool slightly, and poured into a gel mould. Appropriate sized combs were fitted, and the gel was left to set at room temperature for at least 1 hour. The finished gel was placed into an electrophoresis tank, containing 800 ml TAE and 0.0025% ethidium bromide. 2 μl of orange G solution (10 g sucrose, 50mg orange G, 5mg sodium azide in 20 ml H_2O) was added to 12 μl PCR product.

This mix was carefully loaded into the wells in sequence. A 1kb DNA ladder was put into the first well of each lane, so that the size of cDNA products could be determined. The voltage was set to approximately 100mV, and the current applied. The cDNA was left to migrate towards the anode through the agarose gel for an appropriate period (approximately 1 hour), to allow adequate separation of the 100Kb bands of the hyperladder.

3.11: Band detection and semi quantification

Ethidium bromide intercalates between molecules of DNA and is fluorescent under long wave ultra violet (UV) light. This permitted visualisation of the DNA bands. Large gels were divided into six to allow centralisation of groups within the UV imager. They were then exposed to UV and captured digitally using the Alpha-Imager and Alphaease 3.3 software imaging system to allow measurement of band intensity and extrapolation as a semi-quantification of PCR product.

3.12: Statistical analysis

Statistical analysis was performed by entering data initially into Microsoft™ Excel spreadsheets and then uploading this data into SPSS (version 14.0) statistical package software (SPSS inc., Chicago) with appropriate statistical tests applied such as analysis of one way variance (ANOVA) with Bonferonnis' post-tests for intergroup analysis. Bonferonnis' method was chosen as it is a simple method, valid for equal and unequal sample sizes and is considered a powerful method for determining significant difference when the number of groups being compared is small. A value for $P < 0.05$ was regarded as statistically significant. All data are expressed as mean $\pm$ standard error (SE).

Chapter 4: Characterisation of osteoprogenitor cell lines

4.1: Introduction

Bone formation is a complex biological process which progresses through distinctive developmental stages, from commitment of mesenchymal derived cells into the osteoprogenitor lineage, followed by mitotic proliferation, through to expression of the osteoblast phenotype and ultimately as terminally differentiated osteoblasts producing mineralised extracellular matrix (Tonna, 1961; Owenm 1990; Aubin, 1995). Understanding the processes of proliferation and differentiation, in tandem with further comprehension of signalling pathways are fundamental to the understanding of normal osteogenisis and pathological osteogenisis, such as that seen in craniosynostosis. This has been advanced by the development of immortalised cell lines. These overcome the limitations of malignant cell lines, which fail to display the normal coupling of differentiation and growth arrest and of primary cell cultures in which data interpretation can be difficult, due to the presence of various cell populations and tendency to lose osteoblast characteristics following repeated subcultivation (Aronow et al., 1990). Furthermore, stable cell lines provide an unlimited supply of material for study. This latter point is important, as the supply of primary bone from patients suffering from craniosynostosis is limited by the small numerical chance that any given individual mutation would present more than once for surgery to our institution, within the research period.

The MC3T3-E1 cell line, derived from newborn murine calvaria is a well established, stable cell line, which has been shown to display the osteoblast phenotype following repeated passages (Kodama et al., 1981). They proliferate until confluent then differentiate and can be induced to mineralise in a fashion which resembles membranous bone formation (Sudo et al., 1983). This line has been well studied and characterised in terms of phenotype and temporal pattern of expression of bone related proteins. Quarles et al., (1992), showed that sparcely seeded MC3T3-E1 cells undergo marked cell replication without expression of alkaline phosphatase (ALP) or accumulation of significant mineralised extracellular collagenous matrix during days 1-9 in culture. During days 9-16, they undergo osteoblastic differentiation that is characterised by rising ALP activity and osteocalcin (OC) secretion. After day 16, the mineralisation of extracellular matrix marks the final phase of osteoblast phenotypic development (**Fig. 4.1**). This work has been

largely confirmed by others, although the temporal stages and phases of marker expression can be variable depending on seeding density, clonal selection drift and numbers of passages (Choi et al., 1996; Beck et al., 1998; Wenstrup et al., 1996; Peterson 2004). Moreover, within our laboratory (Petoit A., PhD), we have generated an MC3T3-E1 cell line stably expressing the wild type human FGFR2 (WT) and to mimic the craniosynostotic phenotype, FGFR-C278F (C278F), a mutated human FGFR2, found in patients with Crouzon, Pfeiffer and Jackson-Weiss syndromes (Oldridge et al., 1995; Steinberger et al., 1996; Passos-Bueno et al., 1998). In this mutation, the cysteine at position 278 in the third immunoglobulin-like extracellular domain IIIa of FGFR2 is replaced by a phenylalanine, causing constitutive activation of the receptor as described in chapter 1.

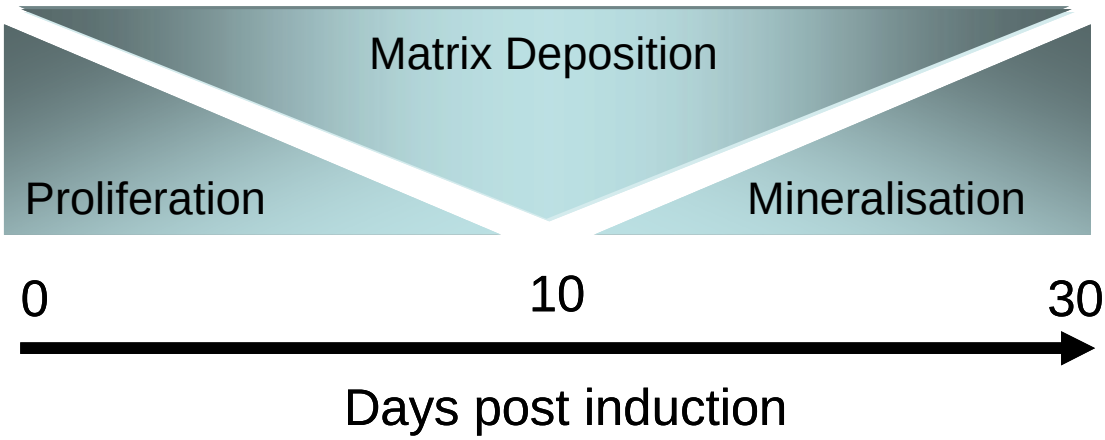


Figure 4.1: Schematic representation of MC3T3-E1 development in culture
Plated osteoblasts initially undergo rapid proliferation with progressive deposition of extracellular matrix, followed by a time dependent decrease in replication with a concomitant increase in extracellular matrix deposition. Finally matrix mineralisation predominates (time-line from Quarles et al., 1992; Choi et al., 1996)

In the context of our long term aims of developing autologous, engineered scaffolds for clinical use, the aim of the work described in this chapter was therefore to better characterise the behaviour of osteoprogenitors carrying this mutation for craniosynostosis and assess their viability as a model for studying the effect of FGFR2 mutations on cellular behaviour and osteogenisis. This would ideally inform the development of potential strategies to modify the behaviour of mutated cells if required.

4.2: Results

The three cell lines were grown under standard culture conditions or in the presence of mineralisation medium. They were assessed morphologically, for their ability to form ossification centres and mineralise and for the expression of cell-cell adhesion molecules, markers of osteodifferentiation and a fibroblast growth factor.

4.2.1: hFGFR2 expression

Prior to assessing the expression of all molecules being investigated, it was necessary to confirm that the cell lines were expressing the hFGFR receptors expected. This was performed by adding a restriction enzyme to the PCR amplified products of hFGFR2. The sequence 5'-GTCTGC-3' is replaced by 5'-GTCTTC-3' in the C278F cells. This is recognised by the BbsI enzyme, creating two fragments. The results confirmed that both the WT and C278F cells produce the hFGFR2 product which is absent in the MC3T3-E1 cells. The restriction site enzyme cut the mutated hFGFR2, but not the wild type receptor product, confirming there was no significant cross contamination of cell lines and that the mutated hFGFR was expressing (**Fig. 4.2**).

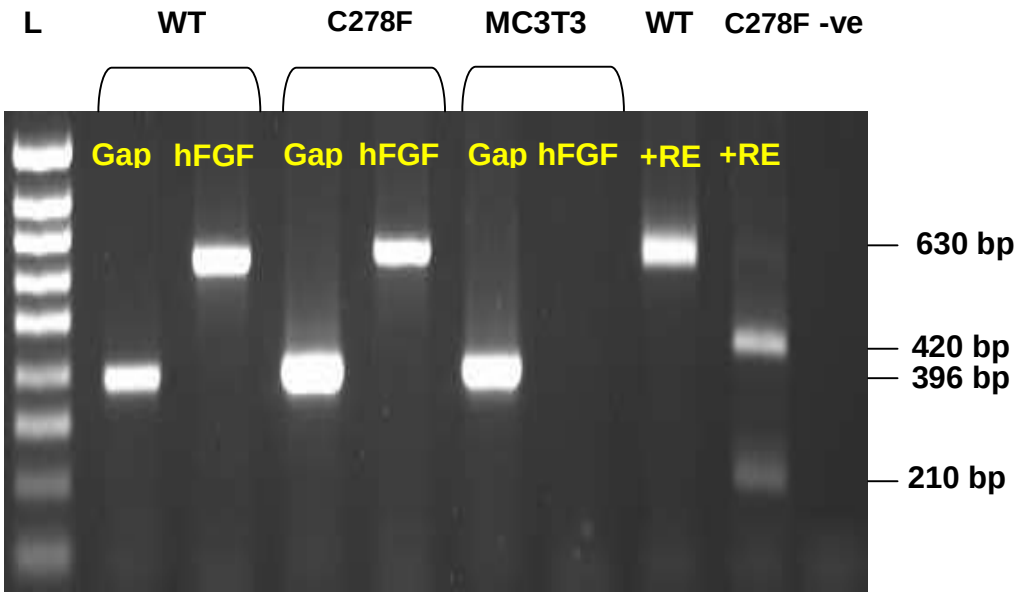


Figure 4.2: Confirmation of hFGFR2 receptor

RT-PCR for FGFR2 was performed on all three cell lines. Expression of hFGFR2 was detected as amplification of a 630 bp product. This was treated with the restriction enzyme BbsI, digesting the product to 420 bp and 210 bp fragments. The samples were run on a 2% agarose gel. **L** = DNA ladder to determine approximate length of amplified cDNA. **RE**= Restriction enzyme BbsI. Negative control (**–ve**) consisted of DEPC water in place of cDNA

4.2.2: Morphology

All cell lines were cultured in normal media as described until confluent and supplemented with ascorbate and ß-glycerol-phoshate thereafter and monitored daily for survival and growth. Initial cultures on plastic revealed that MC3T3-E1 and WT cells appeared to grow well on a medium change regime of every 3rd or 4th day. However, C278F cells did not thrive on this regime. There was notable early acidification of media indicated by a change in media colour. This required a change to an alternate day feeding regime, which was applied for all lines thereafter.

There were marked difference in the gross appearance and attachment behaviour between the lines: C278F cells carrying the craniosynostotic mutation appeared to adhere less well than either the untransfected MC3T3-E1 cells or the WT cells carrying the non-mutated human FGFR2 receptor. MC3T3-E1 and WT cells attached evenly to plastic

within 2-4 hours developing a fibroblastic, elongated morphology, typical of proliferating osteoblasts (**Fig. 4.3 A-B**) In contrast, C278F cells (**Fig. 4.3 C**) attached poorly at first, requiring 4-6 hours before significant attachment was observed. However, they subsequently grew at a rate which appeared similar to the growth of the other lines. This was confirmed by measures of plating efficiency (**Fig. 4.4**).

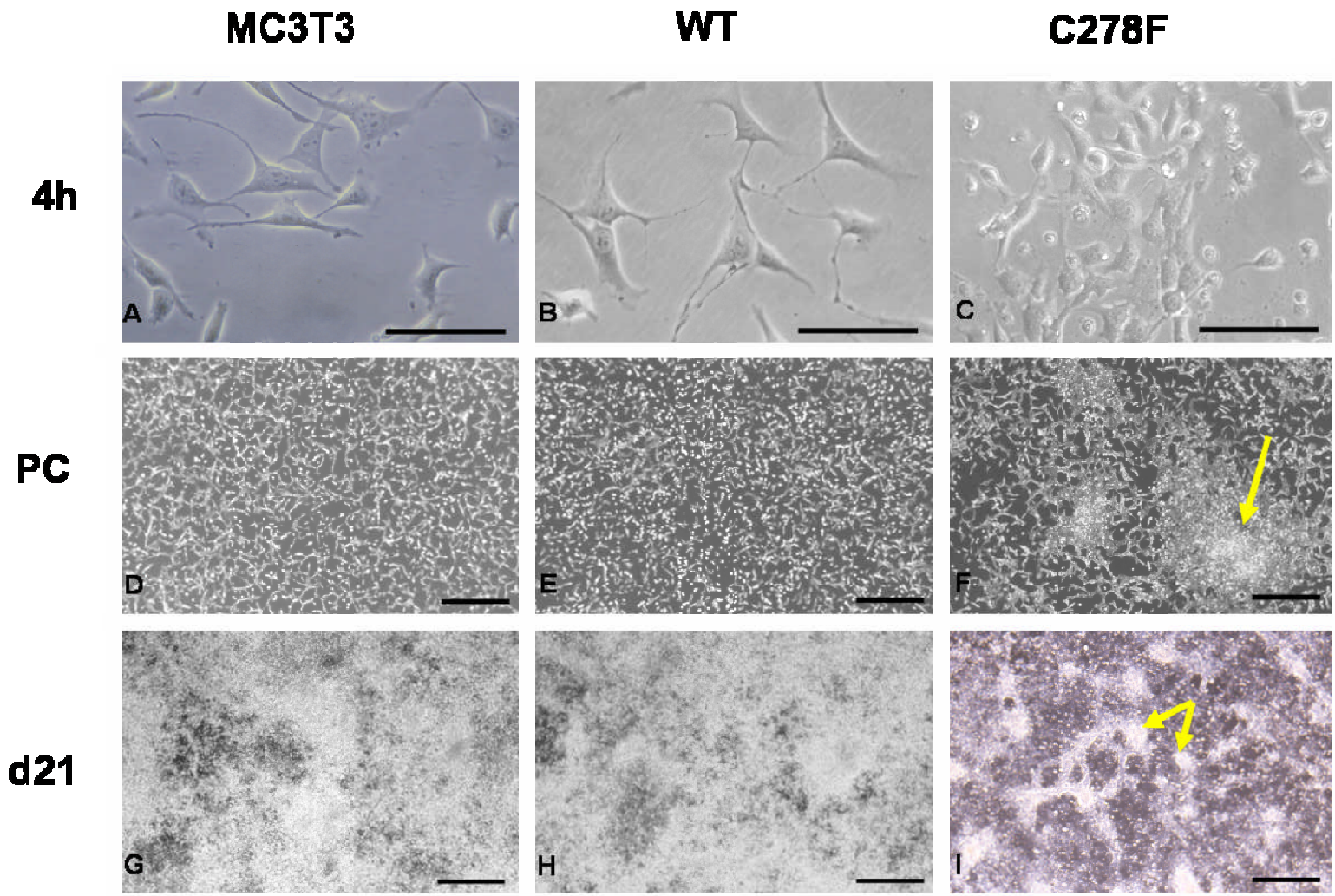
The mutated cells did not develop such an elongated or dendritic morphology and unlike the evenly distributed MC3T3-E1 and WT cells (**Fig. 4.3 D-E**), they formed aggregates (**Fig. 4.3 F**). Further proliferation demonstrated that MC3T3-E1 and WT cells reached confluence after 4-5 days of culture, whereas C278F cells never became fully confluent under normal conditions. There was an apparent high level of cellular death in the C278F cultures, heralded by the vacuolated appearance of cells and subsequent appearance of floating dead cells and cellular debris not seen in the MC3T3-E1 or WT cultures.

C278F cells aggregated and proliferated in distinct condensations which progressed to form multilayered nodules (**Fig. 4.3 I**). Nodular areas developed less obviously in the MC3T3-E1 and WT cultures (**Fig. 4.3 G-H**). During all phases of growth, although more prominently in the differentiating phase, the observed number of dead cells and cellular debris was greatest in the C278F cultures.

When cells were released from plastic, MC3T3-E1 and WT cells detached similarly. However, C278F cells detached more readily after approximately half the exposure period to trypsin-EDTA. This raised the possibility that cell-cell and cell-substrate adhesion molecules may be functioning differently in mutated cells. It was also noted that the mutated cells appeared less tolerant to release from plastic by trypsinisation than the MC3T3-E1 and WT lines, with a visibly higher proportion of cells not surviving the process.

Figure 4.3: Morphology of MC3T3, WT and C278F cells

MC3T3-E1 (**A,D,G**) WT (**B,E,H**) and C278F (**C,F,I**) cells from a second passage post plating were seeded at 12,000 cells/cm^2 and grown in αMEM containing 10% FCS until confluent (designated day 0). Medium was then supplemented with 50µg/ml (final concentration) ascorbate and 10 mM ß-glycerophosphate. Cells were imaged by phase contrast microscopy at 4 hours post plating (**A-C**), Pre-confluence(PC) (**D-F**) and 21 days post confluence (**G-I**). Arrows (**F, I**) indicate areas of nodule formation. Scale bars are 25µm in A-C and 200µm in D


MC3T3
WT
C278F
4h
PC
d21
A
B
C
D
E
F
G
H
I

4.2.3: Plating efficiency

MC3T3-E1 cells showed the most efficient attachment. Mutated cells attached poorly in comparison with MC3T3-E1 and WT cells. This was statistically significant at 1, 2 and 4 hours. However, mutated cells appeared to demonstrate “catch-up” growth when attached, suggesting their proliferative rate may be greater than that of the MC3T3-E1 or WT cells. At 4 and 8 hours for MC3T3-E1 cells and at 8 hours for WT cells, the number of cells attached was greater than 100%, reflecting that these cultures had undergone mitotic expansion beyond losses due to cell death and non attachment.

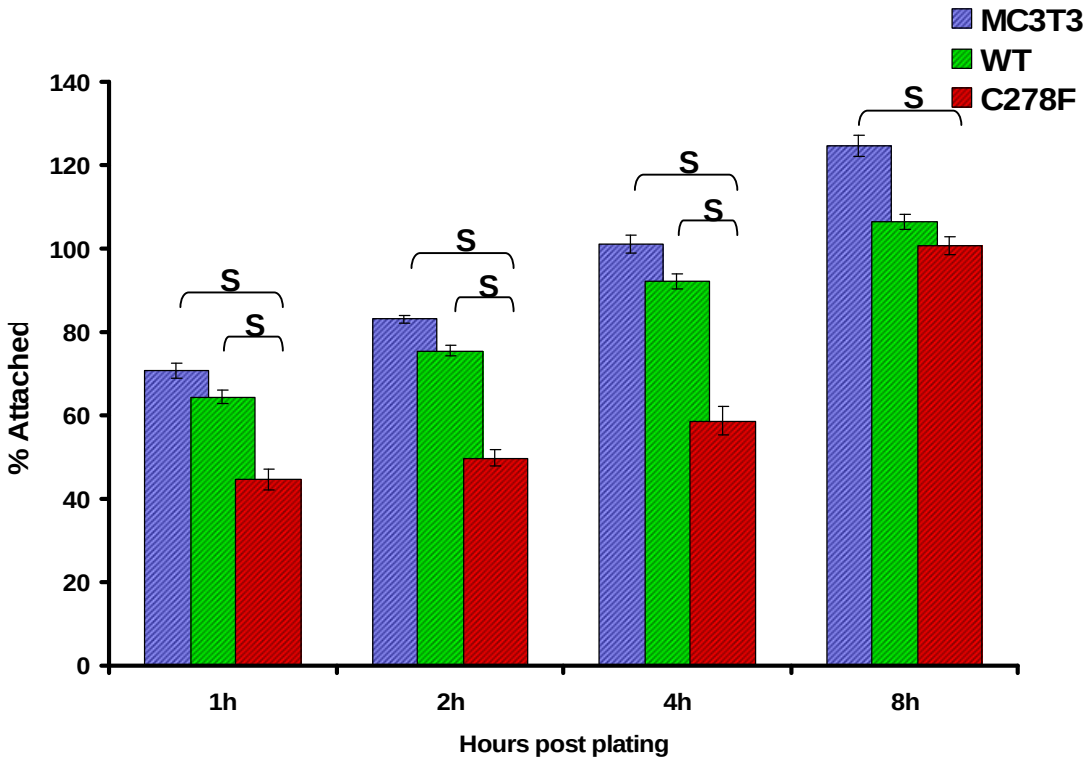


Figure 4.4: Plating efficiency of cell lines

Cells were plated at 12,000/cm^2 in standard medium. Detached cells were counted at 1,2,4 and 8 hours following seeding. Data are expressed as the mean $\pm$ SEM for each group. Statistical differences among treatment groups were evaluated using analysis of variance (ANOVA) and the significance of differences was determined by *post hoc* testing using Bonferroni’s method. **S** = significant ($p < 0.05$ was considered significant)

4.2.4: Cellular Proliferation

Initial observations of the behaviour of the cell lines and indications from measures of plating efficiency, suggested that despite poor early attachment, mutated cells exhibited “catchup” growth.

Studies have identified that FGF/FGFR signaling has profound effects on cellular growth and differentiation and therefore it might be expected that the C278F cells would exhibit altered proliferative characteristics compared to the wild-type.

To study proliferation in the C278 cells, the mitotic index was studied using anti-Histone-H3 antibodies. Monoclonal antibodies directed against histone H3 phosphoserine have been used to visualise mitotic chromosomal condensations to provide staining indices that correlate well with simple mitotic indices (Chadee et al., 1999, Hirata et al., 2004). The phospho-H3 antibody recognises histone H3 after it becomes phosphorylated on serine 10 when the chromosomes condense during prophase, (Strahl & Allis, 2000). Histone H3 remains phosphorylated until telophase, at which time it becomes dephosphorylated by specific phosphatases (Cunningham, 2002).

This study revealed a greater mitotic index for C278F cells (**Fig. 4.5**) which became statistically significant at day 2 (**Fig. 4.6**).

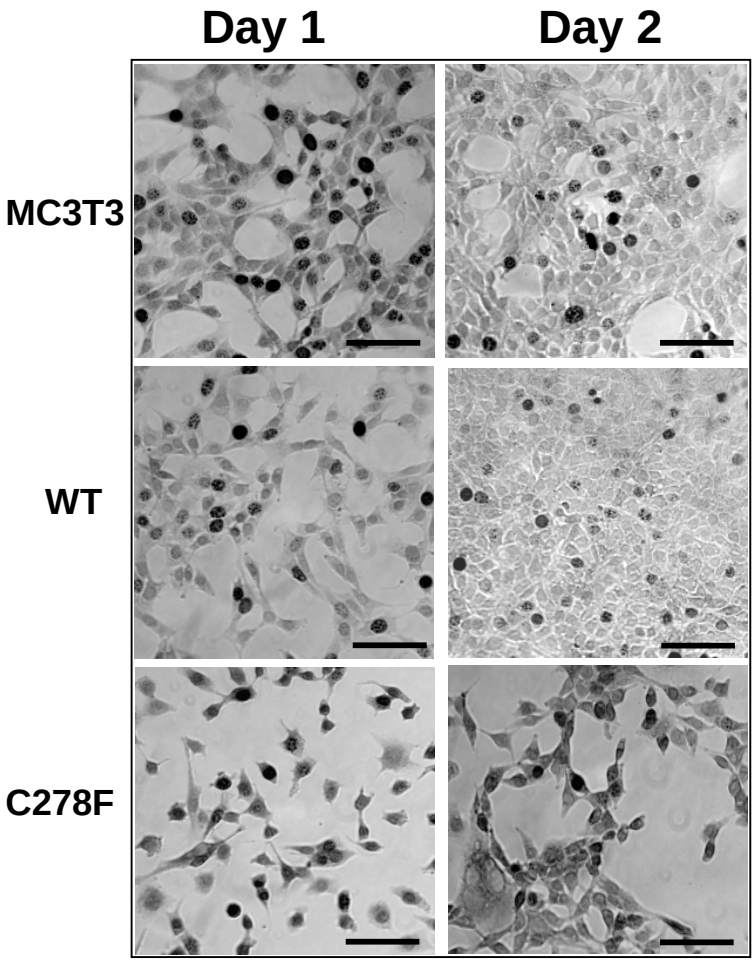
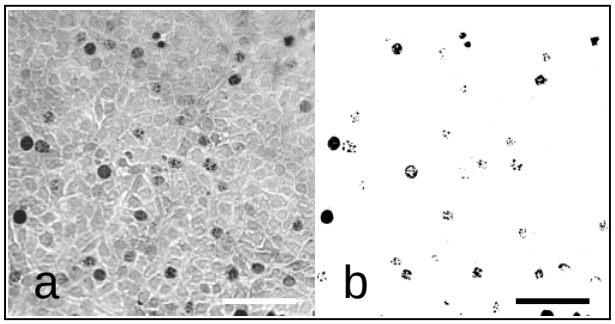


Figure 4.5: Phosphorylated histone 3 (p-H3) immunostaining of osteoblasts
Histone staining of mitotic chromosomal condensations of cell lines at 24 and 48 hours after plating in normal medium at 12,000 cells/cm^2. Four random areas per well (triplicate samples) were counted to give 12 counts for each cell line and time point. A negative control (no primary antibody applied) for each time point was included (not shown). Images obtained by phase contrast microscopy. Scale bars = 50 µm

A



B

Figure 4.6: Measurement of Mitotic Indices for MC3T3-E1, WT and C278F cell lines

A. Example (WT cells at 48hours) of method to count H3 positive cells. All cells (**a**) were counted using Openlab software "click counting" facility. This was repeated (**b**) following adjustment of gain and contrast levels to show only cells demonstrating H3 positivity. Images obtained by phase contrast microscopy. Scale bars = 50µm

B. Mitotic index expressed as % of positive cells/total cell count. Data are expressed as the mean ± SEM for each group (n=12). Statistical differences among treatment groups was evaluated using SPSS (version14.0) (SPSS inc., Chicago, USA) software by analysis of variance (ANOVA) and the significance of differences was determined by *post hoc* testing using Bonferroni's method. **S** = significant ($p < 0.05$ was considered significant)

4.2.5: Matrix mineralisation

The mineralisation of matrix proteins is of fundamental importance in osteogenisis as would be the ability of any potential cell based biomaterials to remineralise in an appropriate manner when implanted.

Quarles et al., (1992) and Choi et al., (1996), demonstrated that the MC3T3-E1 cell line start to produce calcified nodules at approximately day 16 in differentiation culture medium. Osteoblasts carrying mutations in FGFRs appear to mineralise matrix in a manner which is dependent upon the specific mutation involved (Fragale et al., 1999).

Cell cultures were stained for Alizarin-S Red and von Kossa to attempt to reveal areas of matrix mineralisation. In the case of Alizarin, a complex which displays a red/orange birefringence is formed between calcium and Alizarin via its sulphonated and hydroxyl groups. The reaction is not strictly specific for calcium, since magnesium, manganese, barium, strontium, and iron may interfere, but these elements usually do not occur in sufficient concentration to interfere with the staining. Furthermore, calcium-binding proteins and proteoglycans are also detected by this stain.

The von Kossa technique which is widely used, relies on the binding of positively charged silver ions with negatively charged phosphate and carbonate ions which act as a surrogate for calcium. These are observed as black/brown deposits. However, caution should be exercised in interpretation, as the presence of phosphate per se does not necessarily imply the presence of calcium or hydroxyapatite.

At 21 days post confluence, C278F cells were observed showing well developed and discrete calcified nodules (**Fig. 4.7 C,F,I**) whereas much more limited nodule formation and calcification was seen in the MC3T3-E1 (**Fig. 4.7 A,D,G**) and WT (**Fig. 4.7 B,E,H**) cells, which demonstrated a more homogenous pattern of staining. All attempts to show mineralisation at 30 days were unsuccessful, due to detachment of the 30 day cultures during the fixation and staining process.

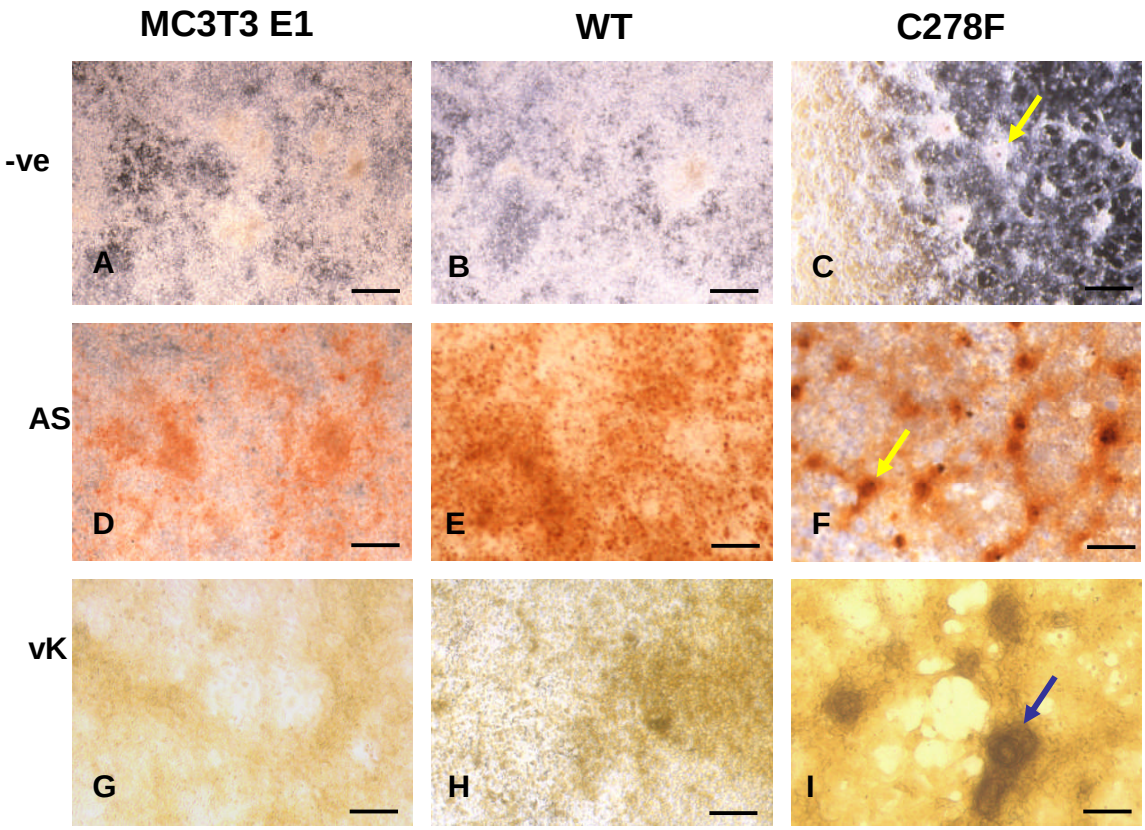


Fig 4.7: Alizarin S Red and von Kossa staining for mineralisation

MC3T3-E1 (**A,D,G**), WT (**B,E,H**) and C278F (**C,F,I**) Cells at 21 days post confluence, unstained (**A-C**), stained with Alizarin (**D-F**) and von Kossa (**G-I**). The mutated cell cultures (**C,F,I**) produced well developed nodules (**Arrows**) which stain for Alizarin S (**AS**) and von Kossa (**vK**) within a background of cells which did not reached full confluence. The MC3T3-E1 (**A,D,G**) and WT (**B,E,H**) cells exhibited very limited development of nodules with a more homogenous pattern of mineralisation. Images obtained by phase contrast microscopy. Scale bars = 200µm

4.2.6: mRNA expression of osteodifferentiation markers

4.2.6.1: Collagen 1a1

All lines expressed collagen1a1 from the pre-confluent stage through to day 30 (**Fig. 4.8**). MC3T3-E1 cells expressed collagen at significantly higher levels from day 5-30. The expression of type I collagen in the C278F cells was not raised in comparison to the WT cells and both were significantly reduced in comparison to untransfected cells. Levels of expression remained at a consistent level throughout the study period in the C278F and WT lines with a tendency to decrease towards day 30 in the mutated cells, although this was not statistically significant. In contrast, levels in the MC3T3-E1 cells increased towards 30 days, although again this was not statistically significant. The change from normal to mineralising medium at day 0 made no obvious difference to levels of expression.

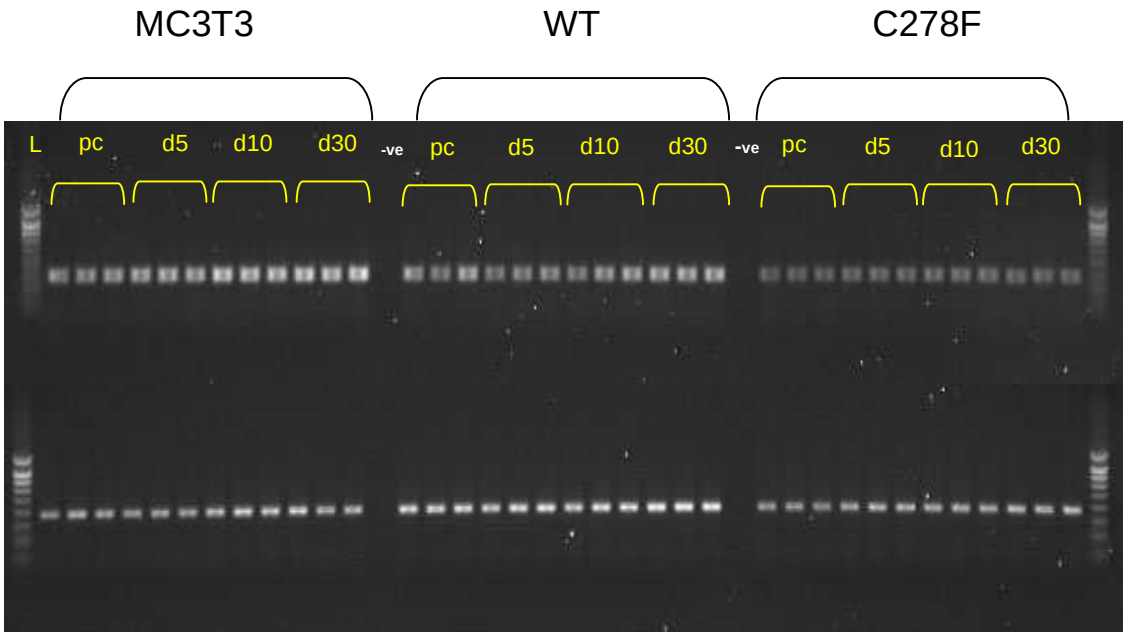


Figure 4.8: RT-PCR of Collagen 1a1 and GapDH expression in MC3T3-E1, WT and C278F cells

Col1a1 (284 bp) and GapDH (396 bp) band detection following agarose gel electrophoresis in buffer containing ethidium bromide. Samples were run from triplicated culture plates on a single large gel. cDNA bands were detected under UV light and intensity was determined with Alphaease (version3.3) (AlphaInnotech Corp., San Leonardo, Ca, USA) software. **L** = DNA ladder. Negative control (**-ve**) consisted of DEPC water in place of a cDNA sample

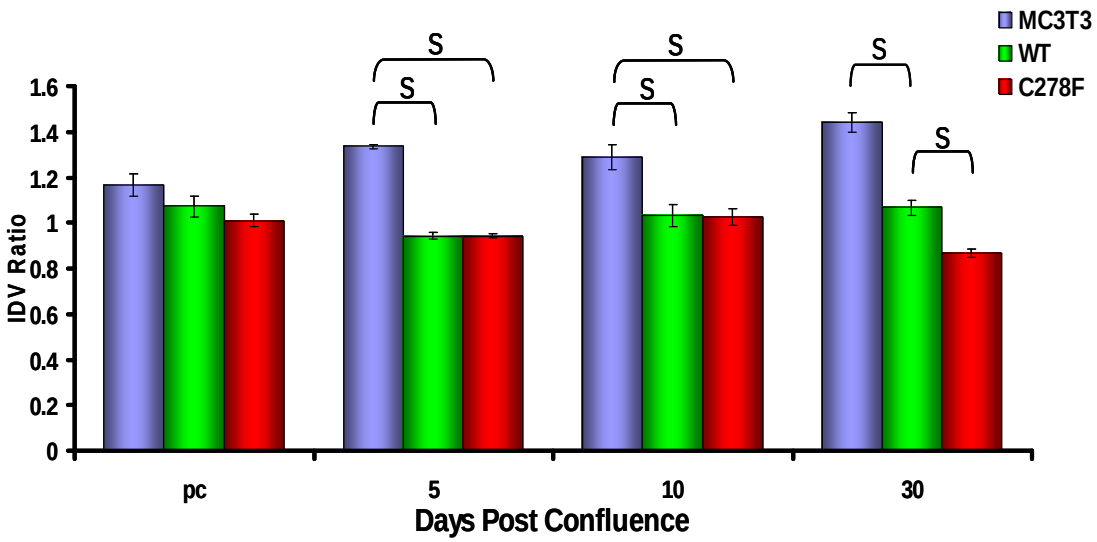


Figure 4.9: Semiquantitative analysis of collagen 1a1 mRNA expression

Band intensity upon UV fluorescence is proportional to the quantity of ethidium bromide which intercalates with the cDNA. This should be proportional to the quantity of RNA at the start of the RT-PCR reaction thereby giving an indication of the levels of cellular RNA expression. However, gel electrophoresis is an end-product technique which cannot be assumed to quantify the PCR product in the exponential phase of amplification. In addition, ethidium bromide is an insensitive technique which is unable to resolve differences lower than approximately 10-fold. Attempts to make the technique as sensitive as possible include visualisation of the bands within the mid-linear range of band intensity as described in section 3.8.3. Band intensity was detected using Alphaease software and expressed as a ratio of Col1/GapDH for each sample point. (**pc**) = pre-confluent. The data are expressed as the mean $\pm$ SEM for each group (n=3). Statistical differences among treatment groups were evaluated by analysis of variance (ANOVA) and the significance of differences between the groups was determined by *post hoc* testing using Bonferroni's method. **S** = significant ($p < 0.05$ was considered significant)

4.2.6.2 Osteocalcin

Osteocalcin expression was significantly higher in the C278F pre-confluent and day 5 post-confluent cultures and decreased with time to levels similar to the MC3T3-E1 and WT lines in which osteoclacin levels showed a non significant tendency to increase after confluence.

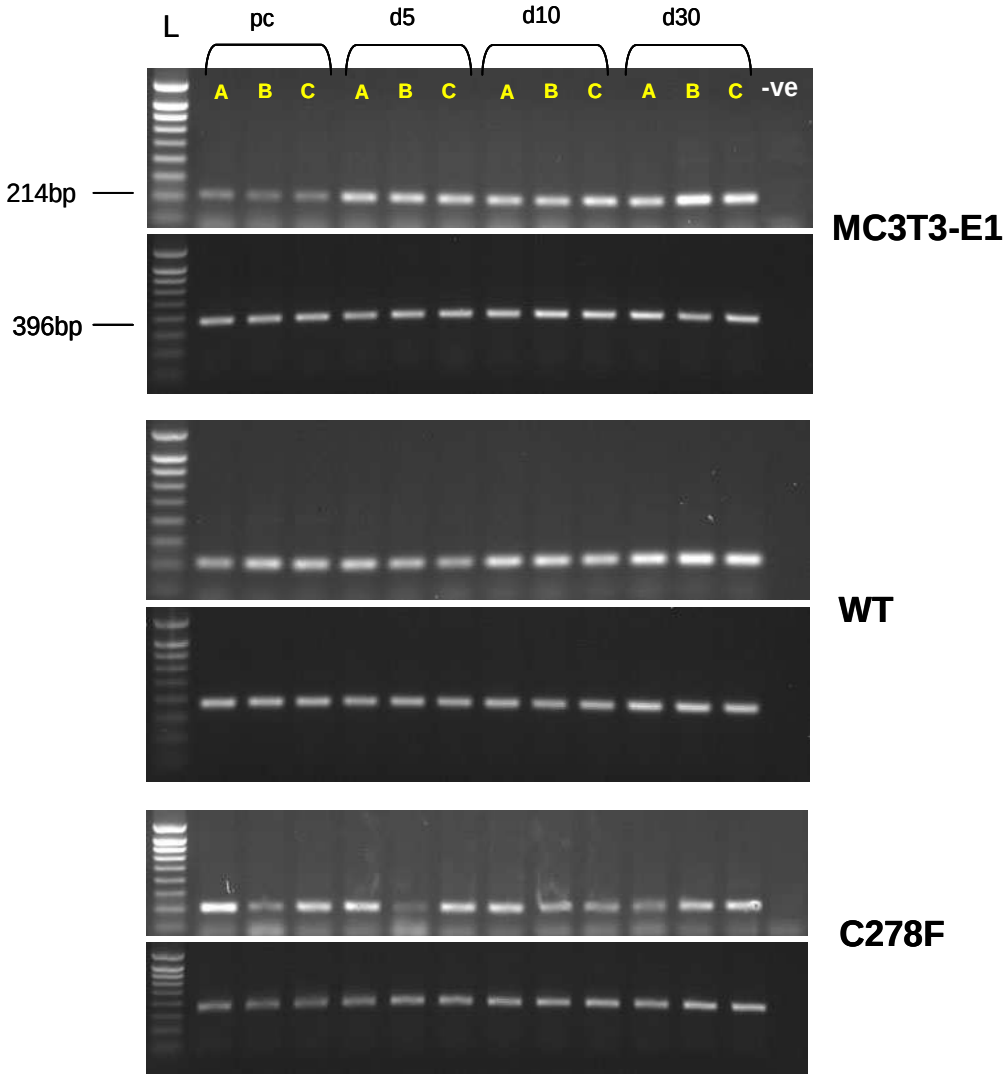


Figure 4.10: RT-PCR of osteocalcin (OC) and GapDH mRNA expression
Osteocalcin (214 bp) and GapDH (396 bp) band detection following agarose gel electrophoresis. Samples for each cell line were run on a single gel and divided for viewing and band intensity reading. **A,B,C** represent separate samples for each line and timepoint. **pc** = pre-confluent, **L** = DNA ladder. Negative control (**-ve**) consisted of DEPC water in place of a cDNA sample

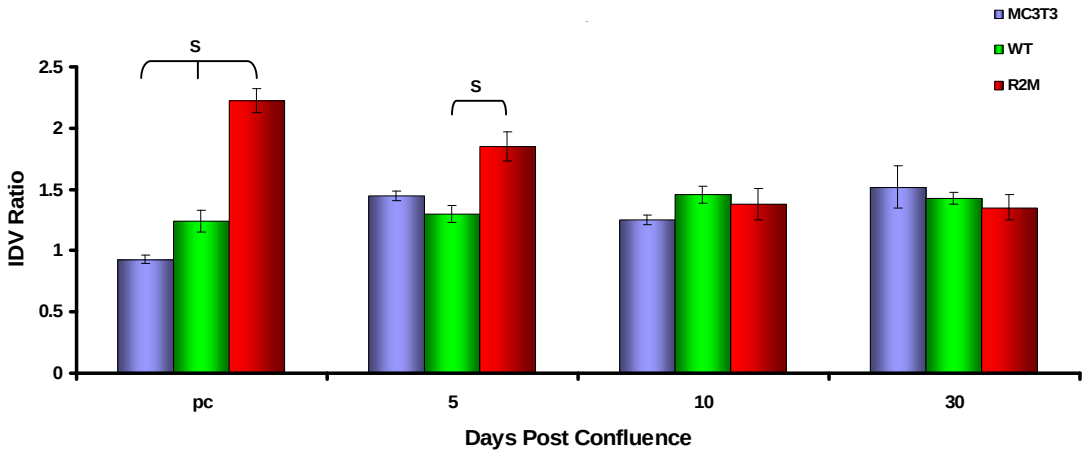


Figure 4.11: Semiquantitative analysis of osteocalcin mRNA expression

RT-PCR analysis of pre-confluent, day 5 – day 30 post-confluent samples of all cell lines. (**pc**) = pre-confluent. Data are expressed as the mean ± SEM for each group (n=3). Statistical differences among treatment groups were evaluated using analysis of variance (ANOVA) and the significance of differences was determined by *post hoc* testing using Bonferroni's method. **S** = significant ($p < 0.05$ was considered significant)

4.2.6.3 Alkaline phosphatase

Alkaline phosphatase was expressed in pre-confluent MC3T3-E1 cells and levels rose towards day 30. Consistently lower levels were seen in WT cells which did not express significantly until day 5. C278F cultures did not express until day 30 and at lower levels than the MC3T3-E1 or WT cultures. Levels were significantly different between all cell lines at days 5-30.

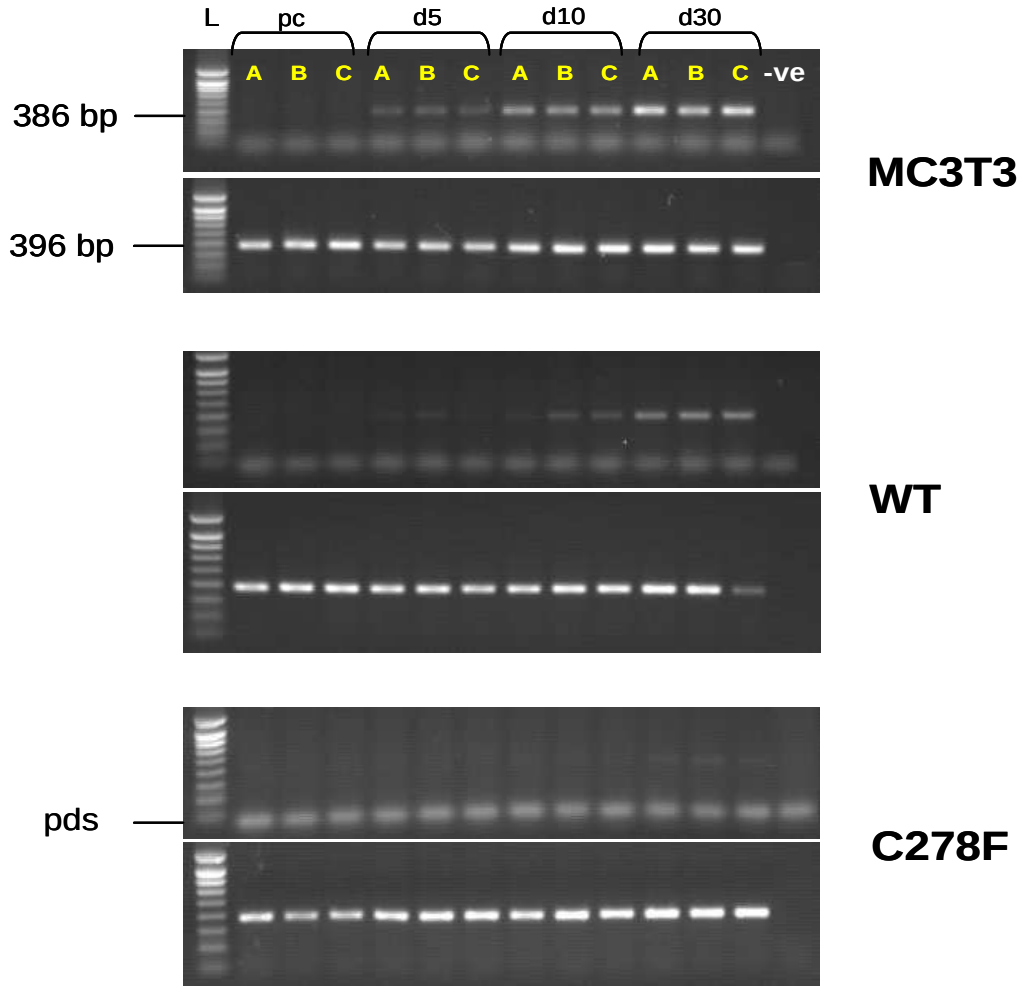


Figure 4.12: RT-PCR of alkaline phosphatase (ALP) and GapDH mRNA expression
Alkaline phosphatase (386 bp) and GapDH (396 bp) band detection following agarose gel electrophoresis. Cell lines were run on separate gels and divided for viewing and band intensity reading. Faint expression observed at day 30 in C278F cells. **pds** = primer dimers. **L** = DNA ladder for determining approximate size of RT-PCR products. Negative control (**-ve**) consisted of DEPC water in place of a cDNA sample

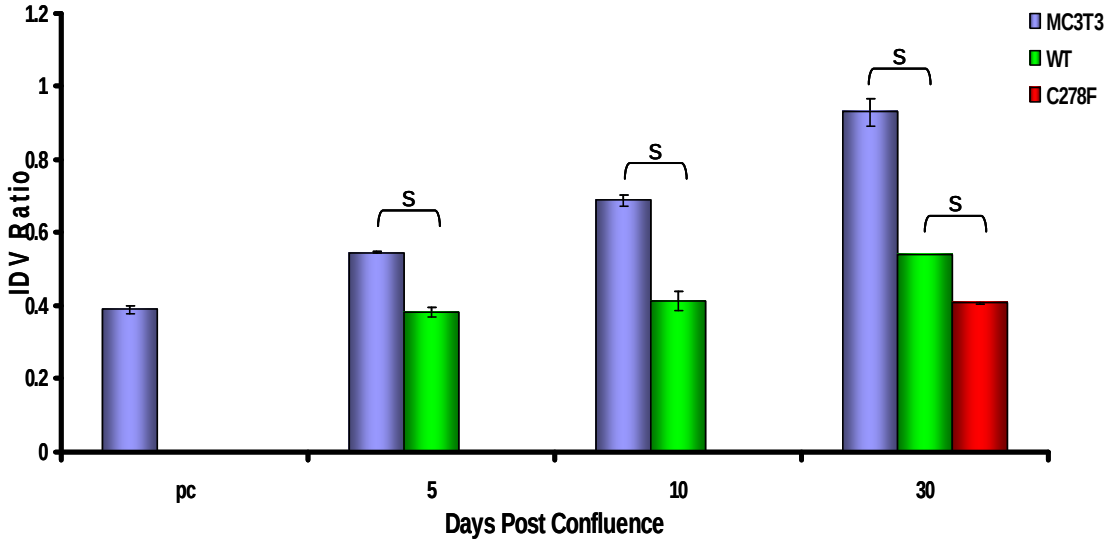


Figure 4.13: Semiquantitative analysis of alkaline phosphatase mRNA expression

RT-PCR analysis of pre-confluent, day 5 - 30 post-confluent samples of all lines. (**pc**) = pre-confluent. Data are expressed as the mean ± SEM for each group (n=3). Statistical differences among treatment groups were evaluated using analysis of variance (ANOVA) and the significance of differences was determined by *post hoc* testing using Bonferroni's method. **S** = significant ($p < 0.05$ was considered significant)

4.2.7: N-Cadherin

MC3T3-E1 cultures showed significantly higher levels of N-Cadherin at all time points. There was no significant difference in the levels of expression between WT and C278F cultures.

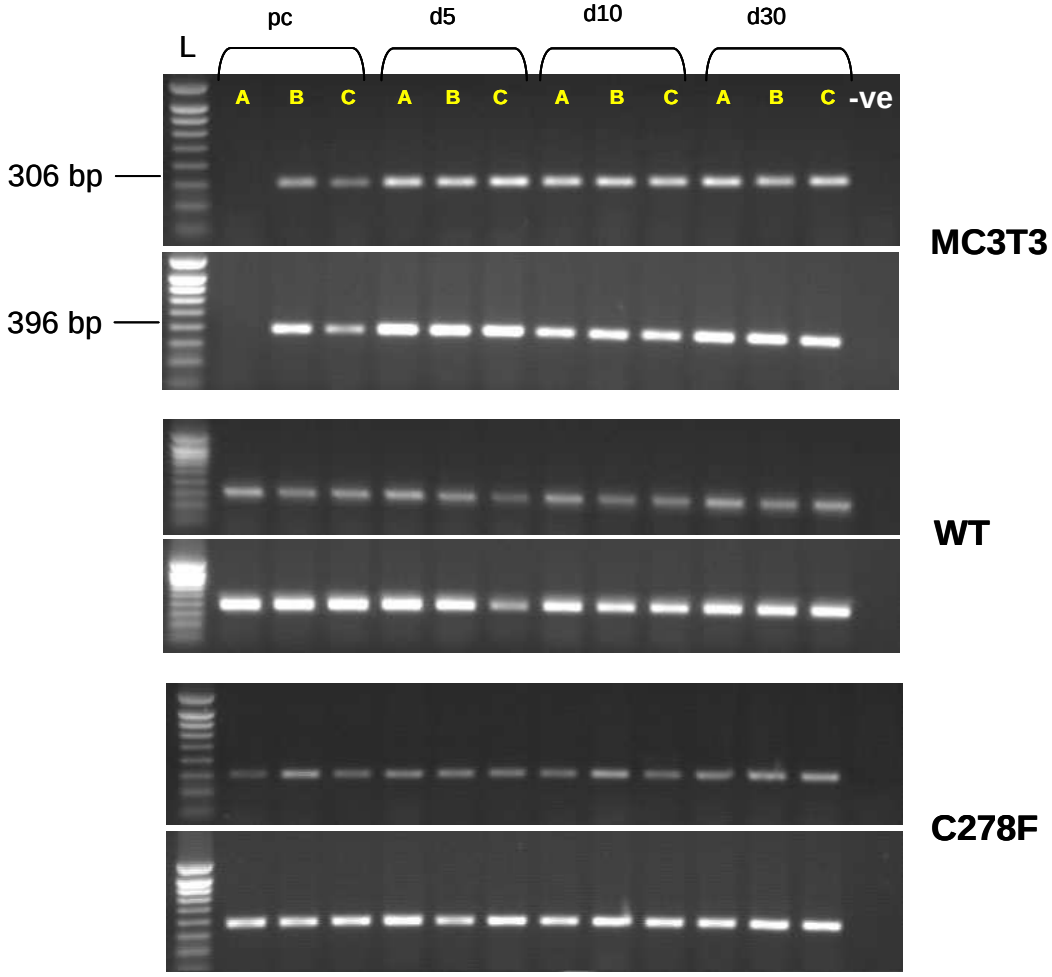


Figure 4.14: RT-PCR of NCadherin (NCad) and GapDH mRNA expression

NCadherin (306 bp) and GapDH (396 bp) band detection following agarose gel electrophoresis. Cell lines run on separate gels and divided for viewing and band intensity measurement. **L** = DNA ladder for determining approximate size of RT-PCR products. Negative control (**-ve**) consisted of DEPC water in place of a cDNA sample

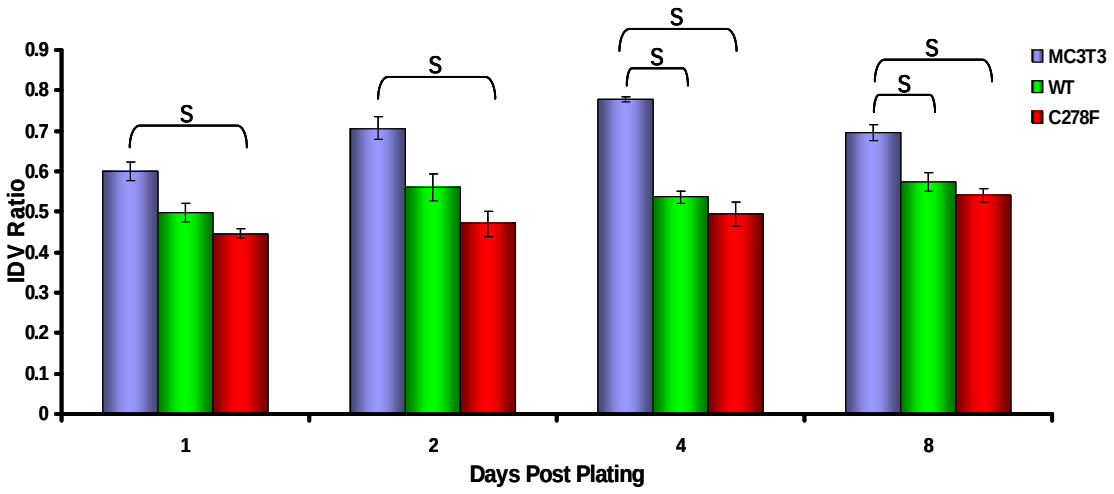


Figure 4.15: Semiquantitative analysis of NCadherin mRNA expression

RT-PCR analysis of day 1 -8 post-plating samples of all lines (day1 – 4 cultures are pre-confluent, day 8 cultures are confluent).

Data are expressed as the mean $\pm$ SEM for each group (n=3). Statistical differences among treatment groups were evaluated using analysis of variance (ANOVA) and the significance of differences was determined by *post hoc* testing using Bonferroni's method.

S = significant ($p < 0.05$ was considered significant)

4.2.8: Connexin 43

Expression in all lines was highest immediately after plating and decreased as the cells became confluent. C278F cultures expressed higher levels than WT at all time points (not statistically significant).

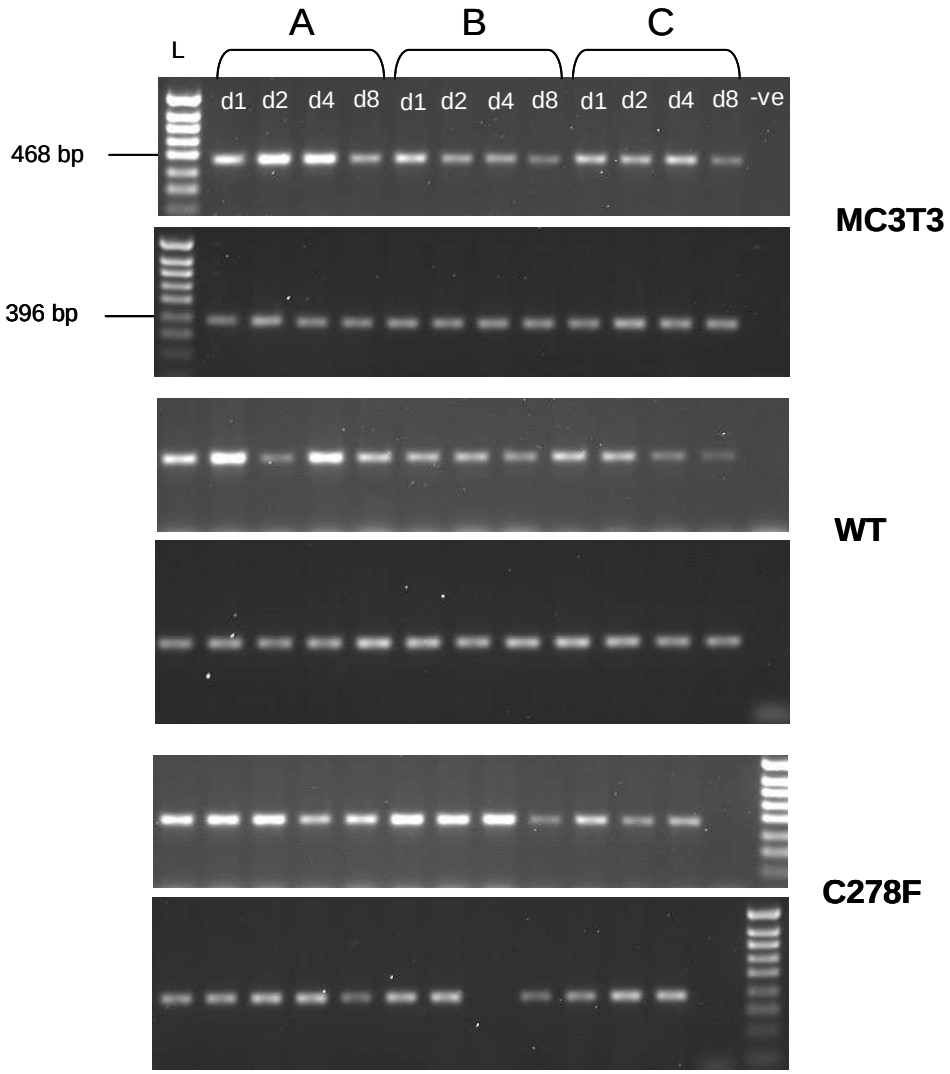


Figure 4.16: RT-PCR of connexin 43 (Cx43) and GapDH mRNA expression

CX43 (468 bp) and GapDH (396 bp) band detection following agarose gel electrophoresis. Cell line samples run on single gels (note the changed well order) and divided for viewing and band intensity measurement. **A,B,C** represent separate samples for each line and timepoint **L** = DNA ladder. Negative control (**-ve**) consisted of DEPC water in place of a cDNA sample

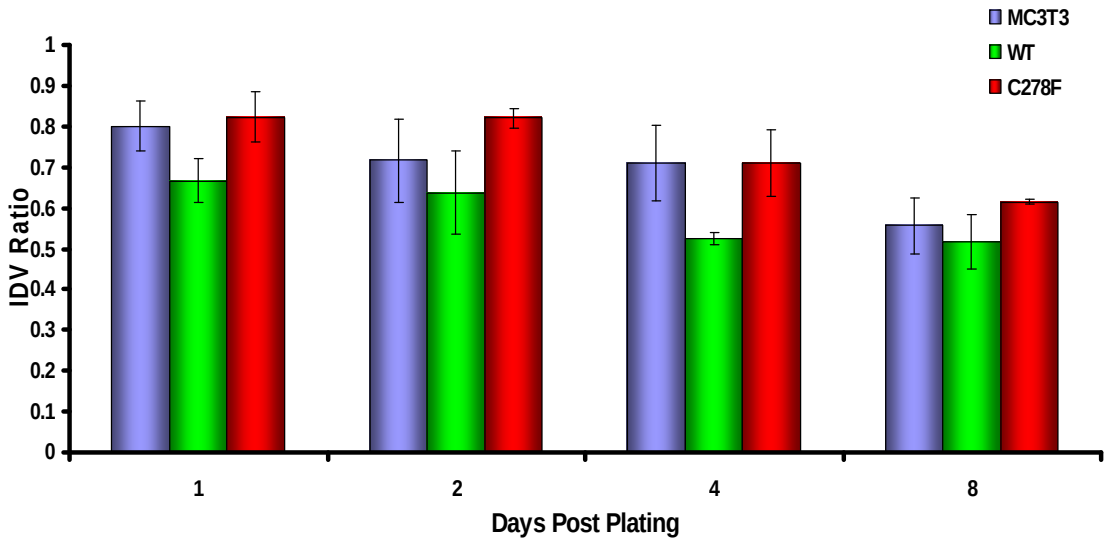


Figure 4.17: Semiquantitative analysis of connexin43 mRNA expression

RT-PCR analysis of day 1 -8 post-plating samples of all lines and their GapDH (day 1 – day 4 cultures are pre-confluent, day 8 cultures are confluent). Data are expressed as the mean ± SEM for each group (n=3). Statistical differences among treatment groups were evaluated using analysis of variance (ANOVA) and the significance of differences was determined by *post hoc* testing using Bonferroni's method. **S** = significant ($p < 0.05$ was considered significant)

4.2.9: FGF18

All lines expressed FGF18 at all time points throughout the culture period. Levels were significantly higher in pre-confluent and day 5 C278F cultures, dropping at day 10 and rising again on day 30. MC3T3-E1 and WT cultures expressed FGF18 at similar levels from pre-confluent to day 10, with levels dropping in these cultures at day 30.

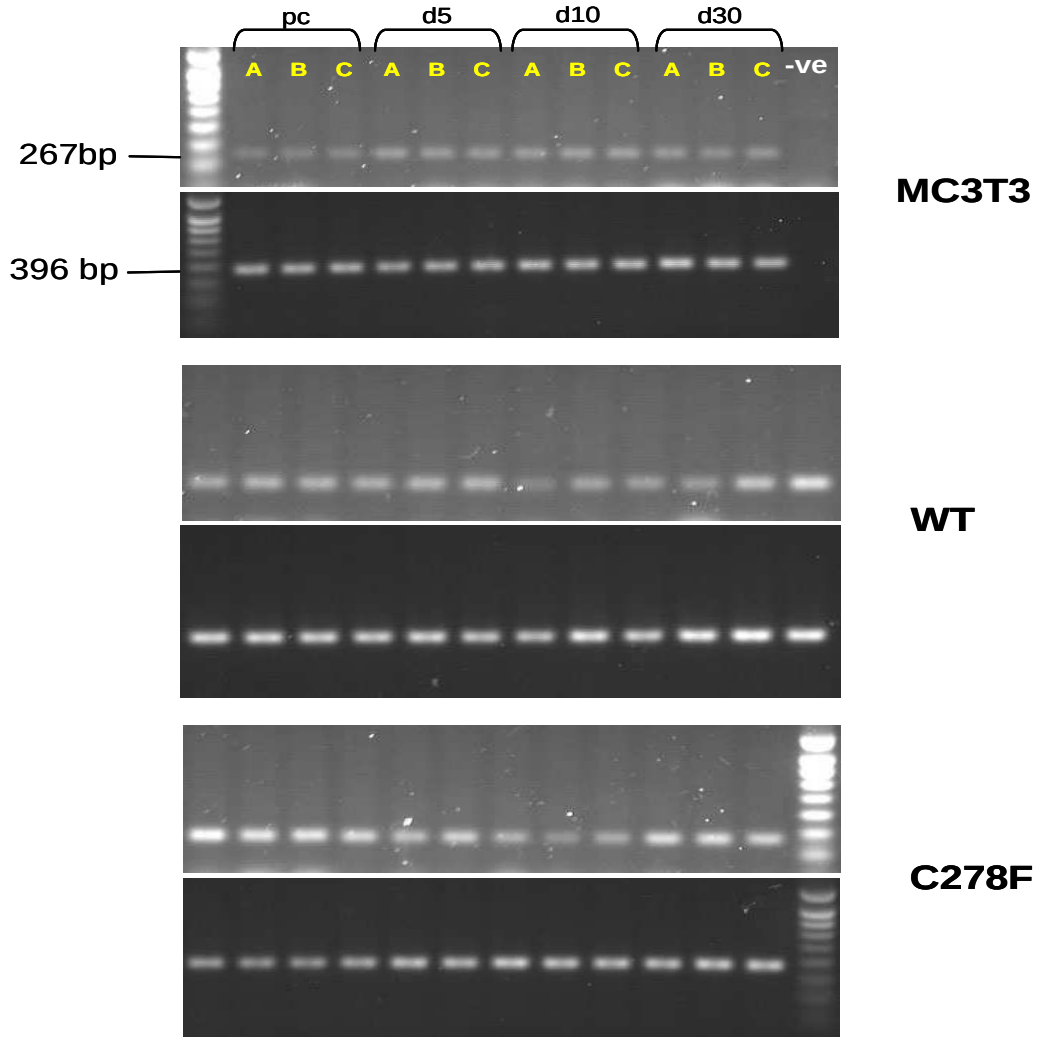


Figure 4.18: RT-PCR of FGF18 and GapDH mRNA expression

FGF18 (267 bp) and GapDH (396 bp) band detection following agarose gel electrophoresis. All samples run on single gel and divided for viewing and band intensity measurement. **L** = DNA. Negative control (**-ve**) consisted of DEPC water in place of a cDNA sample

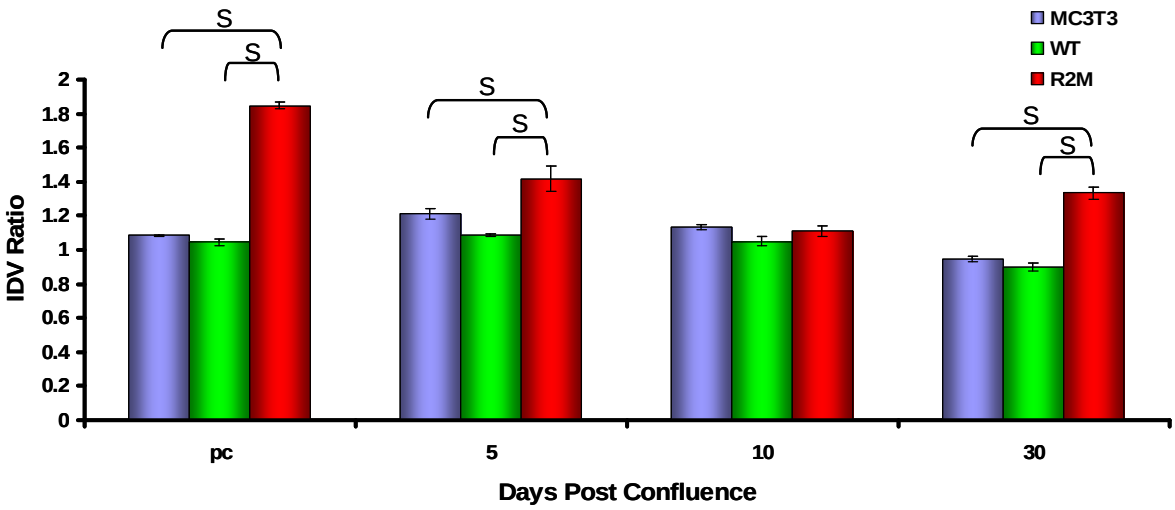


Figure 4.19 : Semiquantitative analysis of FGF18 mRNA expression

RT-PCR analysis of pre-confluent, day 5 - 30 post-confluent samples of all lines and their GapDH. (**pc**) = pre-confluent. Data are expressed as the mean ± SEM for each group (n=3). Statistical differences among treatment groups were evaluated using analysis of variance (ANOVA) and the significance of differences was determined by *post hoc* testing using Bonferroni's method. **S** = significant ($p < 0.05$ was considered significant)

4.3: Discussion

4.3.1: Morphological development of cell lines

Initial observations of cellular growth in culture revealed the C278F cells required a much more frequent feeding regime than non mutated cells. This might be explained by the recent findings of Hatch et al., (2006), who observed significantly higher levels of anaerobic metabolism within COS7 cells transfected with the FGFR-C278 mutation.

Importantly, an obvious difference in the morphology, attachment behaviour, growth and levels of cellular death of the mutated C278F cells compared to the MC3T3-E1 and WT cells was noted. C278F cells initially attached poorly compared to the MC3T3-E1 and WT cells, as confirmed by measures of plating efficiency. When mutated cells attached and began proliferating, their morphology consisted of a more rounded or cuboidal appearance than the other cell lines. Mutated cells did not reach confluence, but formed aggregates early, which developed into multilayered nodules with cultures taking on an overall “woven” appearance. These observations were consistent with the findings of Petiot et al., (2002), who studied the effect of the C278F mutation in premigratory quail chick neural crest cells and in primary human cell cultures carrying different activating mutations of FGFR2 (Lomri et al., 1998; Santos-Ruiz et al., 2007). When cultures were treated with trypsin, mutated cells rounded and detached more quickly and demonstrated a greater tendency to attach to each other.

In considering the possible mechanisms to explain these findings it is worth first discussing the potential influences on cell shape. Two principle models of cellular shape exist. In the first, the cell is a balloon type structure in which the cell membrane influenced by external forces (e.g adjacent cells, matrix), contains a homogenous, viscous cytoplasm (Evans and Yeung 1989; Dong et al., 1991). This fails to explain however, the influence of internal microstructure. Hence, the “tensegrity” model popularised by Ingber, (1993), has become the predominant view with respect to cell shape and movement. In this model, cells generate their own internal tension or “prestress” in the actin filament and intermediate filament cytoskeleton. This is counter-balanced by both internal microtubule struts which withstand compression forces and by external ECM adhesions via cell surface adhesion receptors such as the integrins.

Consistent with the model, it has been shown that forces from the exteracellular environment acting through the cellular adhesion molecules, can be transmitted to the nucleus via the cytoskeleton to influence gene expression (Wozniak et al., 2000; Chen et al., 2001; Jalali et al., 2001; Wang et al., 2001). The mechanotransduction of these signals occurs through focal adhesion proteins such as Vinculin which connect directly to

microfilaments and indirectly to microtubules and intermediate filaments (Alenghat et al., 2000). This in turn activates integrin-associated signaling cascades such as focal adhesion kinase (FAK), extracellular signal-regulated protein kinase (ERK), and G-proteins. (Janmey et al., 1998)

The link between FGFRs and cytoskeletal transduction of signals has not been fully defined. However, Mehta et al., (2001), have documented the p-38(MAPK) dependant formation of actin plaques and cell membrane retraction upon treatment of prostate-DU145 cells with FGF2. More recently, Sandilands et al., (2007), have shown in foetal mouse fibroblasts that ligand-induced activation of FGFRs is dependant on intact actin filaments to allow function of the Src/RhoB endosome shuttle which transfers FGFR from the cytoplasm to the cell membrane. More specifically with relation to craniosynostosis, micro-array studies of pericranial fibroblasts from patients with Crouzon syndrome have demonstrated the down-regulation of Cofilin and Profilin (Carinci et al., 2002). Cofilin is an actin-modulating protein that binds and depolymerises filamentous actin and inhibits the polymerisation of monomeric actin and is involved in the translocation of the actin-cofilin complex from cytoplasm to the nucleus, while Profilin is important for spatially and temporally controlled growth of actin microfilaments. These reports describe important interactions between FGFR signaling and the cytoskeleton to affect cellular shape and function and give further insight into the morphology and behaviour of the C278F cells, suggesting that potential down regulation of these proteins in the mutated cells leads to a decrease in prestress and subsequent rounding of the cells.

The tensegrity model also goes some way to explaining that cellular behaviours, including differentiation, motility, and contractility, can be altered by changing cell shape or ECM rigidity (Ben-Ze'ev et al., 1988; Opas, 1989; Mochitate et al., 1995; Lee et al., 1997; Parker et al., 2002). In general, spread cells grow, retracted cells differentiate and fully round or detached cells undergo apoptosis (Weaver et al., 2002), even when stimulated with optimal levels of growth factors and ECM attachment (Dike et al., 1999). Therefore, these findings may help to explain the observation that the more rounded, less well attached C278F cells had an apparently higher rate of cellular death suggesting that they maybe further down the differentiative path than MC3T3-E1 and WT cells.

4.3.2: Cellular proliferation

Although C278F cells initially attached poorly, they appeared to undergo “catchup” growth upon measures of plating efficiency. This was confirmed by H3 proliferation studies which revealed a greater mitotic index for C278F cells, which became statistically significant at day 2. The finding of differential proliferation between the lines was not altogether surprising as Li et al., (1994), demonstrated that fibroblast proliferation is mediated through FGFR1 and FGFR2. Mansukhani et al., (2000), transfected the OB1 osteoblast cell line with an FGFR2 mutation for Apert syndrome (S252W) and Crouzon syndrome (C342Y) and found that both mutations conferred a two-fold increase in proliferation. Other authors however have reported differently. Lomri et al., (1998), found that osteoblast culture from patients carrying the S252W mutation for Apert syndrome have no alteration in their proliferation rate compared with aged matched controls. Fragale et al., (1999), reported decreased osteoblast proliferation in a patient with Apert syndrome (FGFR2/P253R) and Pfeiffer syndrome (FGFR2/C342R). However, Ratisoontorn et al., (2003), have found that chicken calvarial osteoblasts transfected with the C278F mutation, exhibited significantly increased proliferation.

Ohbayashi et al., (2002), have shown that FGF18 is implicated in the control of osteoblasts proliferation/differentiation and hence the finding in this study that FGF18 is expressed at significantly higher levels within the C278F cells during early proliferation, may further explain the increased levels of proliferation within these cells.

Mutated cells failed to reach confluence prior to developing nodular condensations and despite the apparently higher levels of proliferation in the first two days post-plating, upon prolonged culture, there was no obvious increase in the total biomass of C278F cells when pelleted for splitting or freezing. This would suggest one of two phenomenons: Either, proliferation in this cell line was down-regulated to a greater degree by cellular communication as the cells became more confluent. Alternatively or in addition, C278F cells may have been undergoing a higher level of apoptosis which would have been consistent with the cellular morphology. Indeed, significantly raised levels of TUNEL (a marker of apoptosis) expression in C278F cells were confirmed by a co-worker in our laboratory (Dr Santos-Ruiz, unpublished data). This is consistent with papers in which osteoblasts carrying FGFR2 mutations exhibit increased apoptosis compared to wild type or control populations (Mansukhani et al., 2000; Lemmonier et al., 2001)

Another explanation of this apparently higher level of proliferation relates to the levels of confluence during the study: Despite seeding at the same cellular concentration and because of the previously described attachment behaviour and higher levels of cell death,

the C278F cells were less confluent than the MC3T3-E1 and WT cells at both time points (**Fig. 4.5**). This gives rise to the possibility that measured differences in proliferation may have been due to a relatively lower level of proliferation within the non mutated lines, secondary to the phenomenon of contact inhibition. This may have become apparent by proliferation studies carried out beyond day 2 once C278F cells were denser. An alternative approach would have been to seed at different densities allowing for plating efficiency and early cell death, to provide cell cultures displaying equal confluence for each time point.

4.3.3: Expression of markers of osteodifferentiation and mineralisation

Although pathologically increased levels of type 1 collagen by calvarial osteoblasts has been demonstrated in craniosynostosis associated with mutations of the FGFRs (Bodo et al., 1996; Lomri et al., 2000; Baroni et al., 2002), the expression of type I collagen in the C278F cells was not raised in comparison to the wild type receptor and significantly reduced in comparison to untransfected cells.

Alkaline phosphatase was not expressed in the mutated cells until day 30 and then at significantly lower levels than the untransfected and WT cells, which first expressed in the pre-confluent and day 5 cultures respectively.

Franceschi et al., (1992, 1994), demonstrated that MC3T3-E1 cells expressing low levels of normal type I collagen, expressed lower levels of ALP and OC. In addition, Wenstrup et al., (1996), found that MC3T3-E1 osteoblasts require a normally structured collagen rich matrix for up regulation of ALP activity and that cells with disrupted collagen structure had down-regulation of ALP but up-regulation of OC, a pattern of expression demonstrated in early cultures of C278F cells, possibly suggesting the presence of an abnormal collagen matrix in the mutated cells.

The finding that Col1 and ALP were expressed at generally lower levels in the mutated cells was seemingly inconsistent with previous reports that type1 collagen and ALP are upregulated in FGFR2 mutations in osteoblasts from patients with craniosynostosis, (Lomri et al., 1998; Fragale et al., 1999), although interestingly, the same authors noted that mineralisation was blocked in osteoblasts from patients carrying Crouzon/Pfeiffer mutations, yet accelerated in osteoblasts from patients with Apert mutations (P253R and S252W). It should be recognised however, that interpretation of the effects of FGFR mutations in patients with craniosynostosis are limited by the varying ages of patients and controls. Nevertheless, these findings are largely corroborated by

osteoblast transfection studies; Mansukhani et al., (2000), reported that mutations producing constitutive activation of FGFR2, block ALP expression and mineralisation.

The fact that the C278F cells were forming prominent nodules and mineralising also seemed at odds with poor expression of ALP. However, during the course of this study, Ratisoontorn et al., (2003), reported the downregulation of type 1 collagen and ALP in chick calvarial osteoblasts transfected with the C278F mutation. Furthermore, Wang et al., (1999), who have shown that there is good correlation between mRNA expression and protein transcription of ALP and OC in the MC3T3-E1 cell line, have questioned the correlation between osteodifferentiation markers and mineralisation, with the finding that MC3T3-E1 cells exhibit clonal subpopulations with widely different expression patterns; some subclones mineralised without expressing ALP or OC, while other clonal populations expressed ALP and OC but did not mineralise. In addition, Beck et al., (1998), showed that ALP expression could be dissociated from OC expression and mineralisation in MC3T3-E1 cells by targeting with adenovirus E1A protein.

Osteocalcin is generally reported as the last marker of differentiation to express, typically appearing not before day 7 in MC3T3-E1 cultures and increasing as differentiation advances (Choi et al., 1996; Quarles et al., 1997). The finding that all cells expressed OC from the preconfluent stage with the mutated cells expressing OC maximally at this stage and decreasing with confluence and nodule formation was surprising. However, this was not an isolated finding, as Ratisoontorn et al., (2003), have also reported their osteoblast lines expressing OC from the outset.

Whilst the differences in levels of expression of ALP and OC between the WT and mutated cells are likely, given the differing morphology and adhesion behaviour, to be due directly to downstream effects of the FGFR2 mutation, it is worth considering that they may be affected by one or a combination of factors not directly attributable to the mutation. The cell lines may be at different levels of maturation for a number of reasons including clonal drift into a phenotypically more differentiated cell line (Leis et al., 1997), selection of particular subclones (Wang et al., 1999) or the effects of multiple passaging, an effect which may be under emphasised in osteoblast research according to Chung et al., (1999) and Peterson et al., (2004).

A further consideration which makes comparisons with the findings of other authors difficult, is possibly the effect which different serum concentrations was having on the cultures: The presence and concentration of the numerous inductive, permissive or inhibitory hormones or cytokines will likely influence osteoblast specific gene expression and cell-matrix interactions. Both the qualitative and quantitative size of these effects

cannot be known and true comparison and interpretation would only be possible by the omission of serum from the experiments.

4.3.4: Expression of markers of cell adhesion

The expression of cell-cell adhesion and gap junction molecules was examined based on the difference in morphology and attachment behaviour of the cell lines as previously described. The expression of Cx43 in all lines was highest immediately after plating and decreased as the cells became confluent. C278F cultures expressed consistently higher levels than WT at all time points although this was not statistically significant. This tendency however, may start to explain the clumping and cell-cell attachment behaviour observed in the mutated cells, as cellular coupling has been directly correlated with Cx43 expression in MC3T3-E1 cells (Yamaguchi et al., 1994).

To date, there are no reports of Cx43 expression in either primary or cell line osteoblasts carrying FGFR mutations. The finding by Gramsch et al., (2001), that there is a correlation between Cx43 expression and increased levels of proliferation in the UMR osteosarcoma cell line would also be consistent with Cx43 expression and increased proliferation of the C278F cells, although extrapolation of findings in an osteosarcoma line to an osteoblast line may be problematic.

When N-cadherin expression was looked at, there appeared to be little difference in the expression levels between the wild type and mutated cells which both consistently and significantly expressed N-cadherin at a lower level than the untransfected cells. This is difficult to explain given the inter-cellular clumping behaviour of the cell lines. Moreover, Lemonnier et al., (2001), have reported the up-regulation of N-cadherin in human primary osteoblasts carrying the S252W mutation for Apert syndrome, although, as previously discussed with reference to osteodifferentiation markers, discordant findings for different mutations within the FGFR2 receptors may be anticipated (Lomri et al., 1998). This is not altogether surprising as the mechanism of receptor activation for the C278F Crouzon mutation involves ligand independent constitutive activation whereas the Apert mutations do not induce ligand independent dimerisation.

4.3.5: Expression of FGF18

FGF18 was expressed in all lines from pre-confluent culture until late differentiation at day 30. Levels of expression were similar in the MC3T3-E1 and WT cells at all time points, decreasing marginally towards day 30. However, the level of expression in the mutated cells was significantly raised by a factor of almost two in preconfluent cultures.

These levels subsequently declined at day 5 although remained raised with respect to the WT and MC3T3-E1. At day 10, levels had fallen to those seen in the MC3T3-E1 and WT cultures. Interestingly, transcript levels rose again in the late differentiated phase.

These findings would suggest that C278F cells were producing raised levels of FGF18 in the proliferative phase, creating an autocrine enhanced proliferative drive. As previously alluded to, Ohbayashi et al., (2002), have shown that FGF18 is implicated in stimulation of osteoblast proliferation via ERK activation. It can therefore be postulated that this up-regulation of the expression of FGF18 may be an attempt to regain functionality by a feedback response to down-regulation of the FGFRs, brought about by the constitutively active FGFR2-C278F. This would be consistent with work published after the completion of this study by Hatch et al., (2006), in which MC3T3-E1 cells transfected with the FGFR2-C278F receptor mutation showed increased receptor internalisation and degradation within the Golgi-ER-Complex and decreased surface expression.

The rise in levels at day 30 is more difficult to explain based on our knowledge to date of FGF18 interactions and effects. Possibly a higher level of cellular death within the C278F cells at late maturation, created a phase in which pockets of unoccupied matrix exist, allowing cells to resume proliferation.

Where statistical significance has been demonstrated, this should be read in the context that a Gaussian distribution was assumed for the distribution of data and sample sizes consisted of triplicates. This is small and indeed the smallest sample size which allows an analysis of variance (ANOVA) and intergroup post-hoc testing. This produces large standard errors which may prevent the identification of real as opposed to statistically significant differences. Furthermore, while the Bonferonni method was chosen for measures of significance because it is considered to be a simple method, good for comparing groups of less than approximately five, it should be recognised that it provides conservative measures of p-value which again may fail to demonstrate significant differences.

4.3.6: Summary

The basic morphological development of the three cell lines and the differences due to the mutation in the FGFR2 receptor has been highlighted. I have demonstrated altered adhesion, cell shape and enhanced early proliferation within the mutated cells. The differential expression of osteodifferentiation markers has been semi-quantified as has the expression of the functionally important cell adhesion molecules Connexin-43 and N-cadherin.

The expression of Cx43 and FGF18, a ligand recently implicated in the control of the proliferative-differentiative balance in osteogenisis has been studied for the first time in FGFR2 mutated cells. It is interesting to speculate as to the molecular causes of the altered cellular morphology and why expression of a mutated FGFR2 receptor should affect the shape and attachments of cells both to themselves and tissue culture plastic. As alluded to, there is probably a complex interplay between cellular adhesion molecules, cytoskeleton, and the nucleus which affects cellular integration and function as predicted by the tensegrity model. This may become increasingly obvious and important when investigating the surface form of scaffolds for bioengineering. The obvious difference in attachment behaviour and shape of the mutated cells would justify further analysis of the nature of adhesion interactions between the cells, the ECM and the expression of cytoskeletal structures. This would facilitate the determination of the fundamental relationship between cell adhesion and function and would have been a focus for future work on this cell model.

What these findings in conjunction with a review of the relevant literature highlights, is that it seems difficult to make broad inferences and apply them to specific cell types or mutations. The interaction of cell type, cell age, the multitude of signaling pathways along with the individual culture and experimental conditions, allow for only limited conclusions to be drawn from any particular set of findings. For example, the precise role which each single component of the extracellular matrix plays in determining the phenotype of any particular craniofacial syndrome is not at all clear.

Having described qualitatively and semi-quantitatively the difference in morphology and behaviour which the FGFR2-C278F(C278F) mutation was eliciting, It seemed relevant to attempt to modify or “rescue” the phenotype of the mutated cell line, to one which more resembled cells not carrying the mutation, with particular emphasis on facilitating maximal proliferative expansion. This would be a potential strategy for utilising mutated, autologous osteoprogenitors for bone engineering. Specifically, it would be informative to explore the effect of FGF18 treatment given the significantly raised levels of mRNA expression within the C278F cell line and the potential importance of this FGF raised in the recent literature.

Chapter 5: Modulation of osteoblast behaviour

5.1: Introduction

In chapter 4, phenotypic study of the cell lines, revealed that in comparison to WT and untransfected MC3T3-E1 cells, FGFR2-C278F(C278F) mutated cells appeared morphologically and behaviourally different. They demonstrated a more rounded/cuboidal appearance when attached to plastic with less well developed filopodia. Attachment behaviour was also markedly different, with C278F cells taking longer to attach to culture plastic and attaching in a manner which allowed a more rapid release by trypsin. Upon attachment mutated cells attached in clusters which subsequently took on a nodular appearance before they were able to reach full confluence. Proliferation studies revealed that once attached the mutated cells demonstrated an initially higher mitotic rate in the early pre-confluent stages of growth. Throughout all culture periods and conditions, the mutated cells subjectively demonstrated a higher proportion of cell death, which was consistent with unpublished data from our laboratory (Dr. Santos-Ruiz), confirming this mutation exhibited increased levels of apoptosis in standard culture conditions. While the expression of mRNA for osteodifferentiation proteins revealed that the appropriate markers were expressed, the temporo-spacial expression was different from that which would have been expected in cells expressing in the accepted progression along the osteoblast lineage. Specifically, the expression of high levels of osteocalcin in pre confluent cultures before the development of significant nodules and the subsequent falling levels were contrary to what was anticipated in osteoblasts (Aubin et al., 1995; Liu et al., 2003).

In considering development of bio-composites for bone engineering, the use of osteoprogenitors from patients with syndromic craniosynostosis and the ability to optimally expand them on a scaffold prior to terminal differentiation and mineralisation would theoretically be an ideal manipulation.

Fibroblast growth factor signalling plays an important role in cranial bone development and osteoblast function as alluded to in chapter 1. The biological effects of FGFs depend on stage of growth, and in vitro studies have shown that FGF1, FGF2, FGF4 and FGF18 can potentiate the growth of proliferating calvarial osteoblasts (Canalis et al., 1988; Tang et al., 1996; Hurley et al., 1995; Shimoaka et al., 2002) but not mature or differentiated osteoblasts (Mansukhani et al., 2000).

Studies within our laboratory have confirmed that endogenous levels of FGF2 may constitute a mechanism to control proliferation in a concentration dependent manner (Moore et al., 2002) and analysis of FGF18 null mice has revealed a decrease in proliferation of osteogenic mesenchymal cells associated with delayed suture closure (Ohbayashi et al., 2002).

Differentiation also seems to be under the control of FGFs, as prolonged treatment with FGF2 promotes osteocalcin transcription and mineralisation in rodent and human osteoblasts (Newberry et al., 1998; Debiais et al., 1998). FGF18 inhibited differentiation and matrix synthesis in MC3T3 cells (Shimoaka et al., 2002). Proliferation and terminal differentiation of calvarial osteoblasts was decreased in FGF18 null mice (Ohbayashi et al., 2002) with a reduction in osteocalcin and osteopontin expression (Liu et al., 2002). In addition, apoptosis is influenced by FGF signalling, which again seems dependent on the stage of osteoblast differentiation. This is important as apoptosis is known to be a fundamental controller of osteoblast longevity and osteogenisis (Manolagas, 2000).

FGF2 treatment of neonatal mouse calvarial osteoblasts protected cells from apoptosis when starved of serum (Hill et al., 1997), whereas FGF1 treatment induced apoptosis in more differentiated osteoblasts and over-expression of FGF2 signalling in transgenic mice led to increased apoptosis in mouse calvaria (Mansukhani et al., 2000; Lemmonier et al., 2001). Mansukhani et al., (2000), also noted similar patterns of proliferation and apoptosis in osteoblasts constitutively activated by FGFR mutations. Interestingly, the effect of exogenous FGF treatment on cells carrying activating mutations for FGFRs has not been studied to a large extent, with only one report by Mansukhani et al., (2000), finding that OB1 osteoblasts transfected with an Apert FGFR2/S252W mutation, demonstrated a 22-54% increase in proliferation upon FGF1 treatment. Crouzon FGFR2/C342Y cells demonstrated only a minimal increase in proliferation upon treatment. FGF1 treatment inhibited osteodifferentiation in these osteoblasts with no quantifiable difference between the groups. Treatment of differentiating, non-mutated OB1 cells with FGF1 induced apoptosis to levels seen in untreated mutated cells.

Thus, FGF signaling of immature osteoblasts appears to reduce apoptosis and increase proliferation and cell numbers, whereas later stage signaling promotes apoptosis. Hence this apparently opposing effect is likely to be an important control mechanism in osteogenisis.

Taken together, along with the findings of Shimoaka et al., (2002), who reported that the mitogenic effects of FGF18 on osteoblasts were as strong as FGF2; the finding in chapter 4 that FGF18 was expressed at significantly higher levels in early and late cultures

of C278F cells, combined with the recent literature suggesting an important role for FGF18 in the control of proliferation/differentiation, directed the study towards examination of the effect of FGF18 treatment on the cell lines.

5.2: Foetal calf serum (FCS)

As seen in chapter 4, the growth of the C278F mutated cells relied on culture medium change more frequently than that required for the WT and untransfected cells. Likewise, the literature reviewed suggests that the concentration of serum in which cells are cultured, influences the response to growth factor treatment (Sachs et al., 1982; Allen et al., 1983). Foetal calf serum contains a variety of polypeptides that may "attach" to cells, inducing significant metabolic/morphologic changes. For example, bone marrow derived mesenchymal progenitor cells show a different behaviour in culture performed in the presence of FCS with or without heat inactivation (Bruinink et al., 2004). It is known that deprivation of serum or growth factors from the medium causes apoptotic death of cultured cells. *Myc* and *p53* appear to be involved in the mechanism underlying such apoptotic cell death, but the precise mechanism is still unknown (Packham et al., 1995; Elliott et al., 1995). Components of serum, such as growth factors other than FGFs highlighted previously, can cause attenuation of apoptosis in cell cultures, for example, epidermal growth factor (EGF) has been shown to protect cultured cells against apoptosis induced by serum deprivation (Tong et al.,, 1999). In addition, increasing MC3T3-E1 cell number has been shown to be linearly proportional to serum concentration up-to 4 days in culture (Masayuki et al., 2000) and the effect of exogenous FGF2 treatment on MC3T3-E1 cells is dependant on serum concentration. It was necessary therefore, to ascertain the lowest concentration of FCS which would sustain the cells during the treatment period and avoid the masking of effects solely due to FGF18.

5.3: Results

5.3.1: Assessment of FGF18 concentration effect

In the first instance, the study observed the effects of FGF18 treatment on the cell lines and established that the concentrations used, were in the correct range to observe an effect. These initial doses were chosen based on the findings of Shimoaka et al., (2002). Initial studies were carried out in 10% serum in keeping with the experiments in chapter 4, in order that any observed differences could be attributed to the FGF18 treatment.

Cellular behaviour observed in culture is shown in figure 5.1**.** MC3T3-E1 cells and WT cells just reached monolayer confluence at 48 hours post plating without treatment (**Fig. 5.1 A,B**), whereas C278F cells displayed their typical non confluent appearance (**Fig. 5.1 C**) as observed in chapter 4. Cells treated for 36 hours with FGF18 at [10^{-10} M], particularly when observed directly through the microscope, appeared more densely packed in the case of the MC3T3-E1 and WT lines (**Fig. 5.1 D,E**).

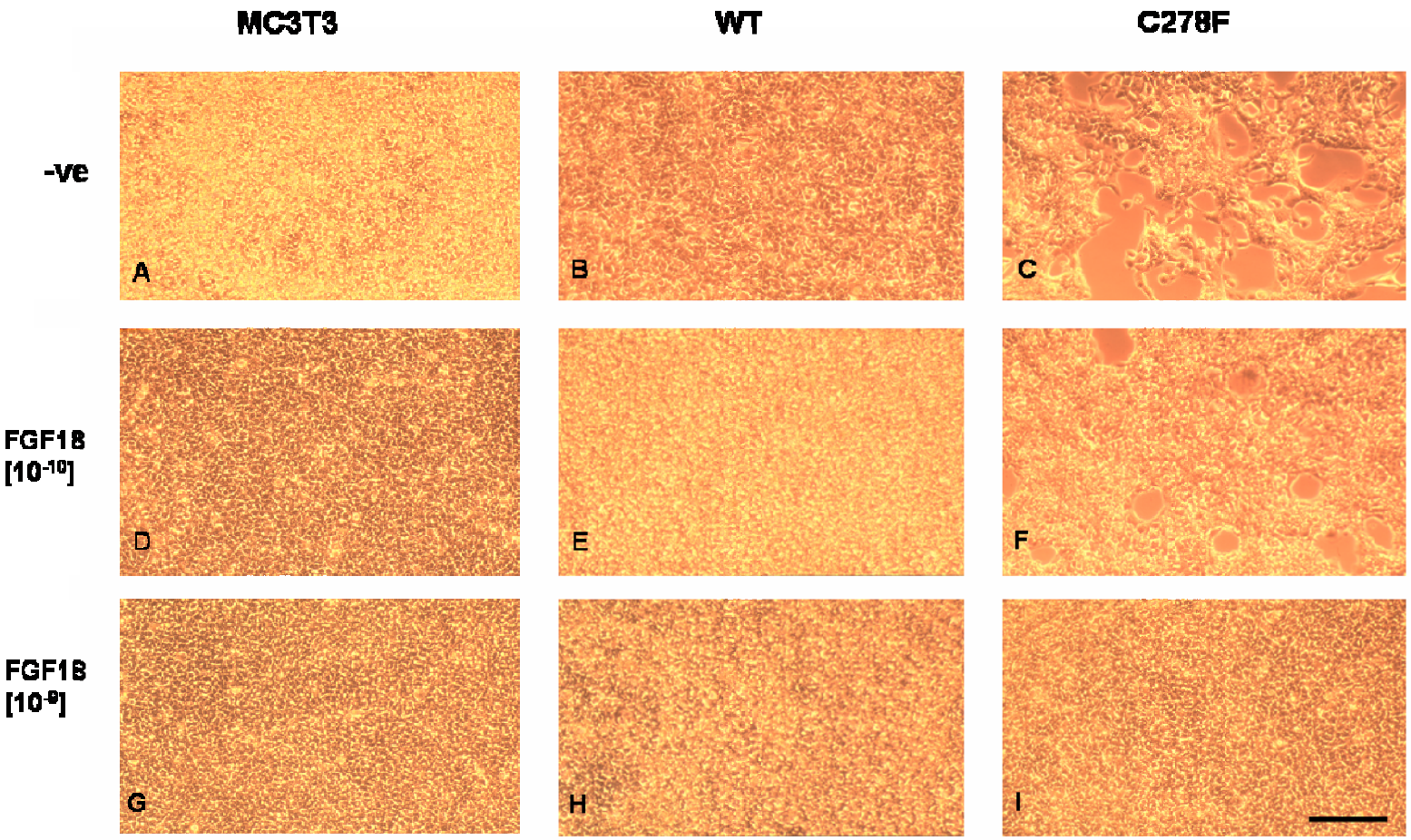
The mutated cells took on a different morphology to that seen in standard culture when treated with FGF18 at [10^{-10} M]: Whilst still not fully confluent there were fewer and smaller voids on the culture dish (**Fig. 5.1 F**), indicating that the treatment had probably increased the cell population and/or altered cellular motility and attachment. When treated with a ten fold higher concentration of FGF18 [10^{-9} M], there was a multilayered appearance and increase in cellular density in the MC3T3-E1 and WT lines (**Fig. 5.1 G,H**), compared to cells treated with FGF18 [10^{-10} M].

Treatment of the mutated cell line with FGF18 [10^{-9} M] (**Fig. 5.1 I**), produced a surprising result in that the appearance of the cultures was indistinguishable from the untransfected and wild-type cell lines. Full confluence had been achieved and there was no evidence of clustering behaviour.

These initial observations appeared to be consistent with the findings of Shimoaka et al., (2002), with respect to the MC3T3-E1 cells. Thus, treatment of the mutated C278F cells with FGF18 altered the characteristics of the line to one which took on the appearance more in keeping with the behaviour of the untransfected and WT cells. This observation suggested that FGF18 treatment might be a candidate for “rescuing” the behaviour of mutated cells in any potential bone engineering application.

Figure 5.1: Treatment of osteoprogenitor lines with FGF18 for 2 Days

Cultures were plated at 12,000 cells/cm2 in standard medium containing 10% FCS. Medium was replaced after 12 hours with standard medium (**A-C**), standard medium supplemented with FGF18[10^{-10}M] (**D-F**) or standard medium supplemented with FGF18[10^{-9} M] (**G-I**). Cultures were imaged at 48 hours following the commencement of treatment. Medium was changed every 24 hours. MC3T3-E1 (**A,D,G**) and WT (**B,E,H**) cells have just reached confluence without treatment(**A,B**) . Treated cells are more densely packed and start to appear multilayered (**D,G** and **E,H**). C278F cells(**C,F,I**) are partially confluent in clusters without treatment (**C**), almost fully confluent with FGF18 [10^{-10} M] (**F**) and fully confluent when treated with FGF18 [10-9] (**I**). Images obtained by phase contrast microscopy. Scale bar = 200 µm


MC3T3
WT
C278F
-ve
FGF18 [10-10]
FGF18 [10-9]
A
B
C
D
E
F
G
H
I

5.3.2: Effects of foetal calf serum

The effect of foetal calf serum (FCS) concentration on the cell lines prior to quantification of the FGF18 effects was studied. The concentration of FGF18 chosen was [10^{-9} M] as this had appeared to "normalise" the proliferation of the mutated cells in section 5.3.1.

MC3T3-E1, WT and C278F cells were observed at 24 and 72 hours with or without the addition of FGF18 [10^{-9} M] in the presence of 10%, 1% or no FCS. The results are shown in **Figure 5.2.- 5.7.** At 24 hours post treatment the MC3T3-E1 cells grown without FGF18 supplementation (**Fig. 5.2 B,D,F**) looked unstressed and showing progressive confluence from no FCS (**Fig. 5.2 B**) to the 10% FCS (**Fig. 5.2 F**) which were near full confluence. When cells were treated with FGF18 (**Fig. 5.2 A,C,E**), the cells growing without FCS (**Fig. 5.2 A**) appeared to be more confluent than the equivalent cells without FGF18. However, they also appeared more "stressed" with a greater number of dead cells suggested by the pattern of light diffraction. The cells growing in 1% FCS (**Fig. 5.2 C**) were fully confluent although once again there was a suggestion that a greater degree of cell death had occurred. Cells grown in 10% FCS (**Fig. 5.2 E**) were fully confluent and looked healthy.

When observed at 72 hours, the MC3T3-E1 cells growing in 1% and 10% FCS were fully confluent with (**Fig. 5.3 c,e**) and without (**Fig. 5.3 d,f**) FGF18 supplementation. Cells grown without FCS and without FGF18 (**Fig. 5.3 b**) looked healthy but still not fully confluent. Whereas cells grown without FCS but with FGF18 (**Fig. 5.3 a**) had changed very little from the 24 hour appearance, looking non confluent and demonstrating signs of stress and cellular death.

WT cells at 24 hours post treatment grown without FGF18 supplementation (**Fig. 5.4 B,D,F**) looked unstressed and showed progressive confluence from no FCS (**Fig. 5.4 B**) to just fully confluent in the 10% FCS condition. When cells were treated with FGF18 (**Fig. 5.4 A,C,E**), cells growing without FCS (**Fig. 5.4 A**) appeared to be undergoing a high level of cell death and appeared much less healthy than in the equivalent group without FGF18. This appearance was significantly improved when grown in 1% (**Fig. 5.4 C**) and 10% (**Fig. 5.4 E**) FCS which were near confluence and confluent respectively. At 72 hours without FCS or growth factor (**Fig. 5.5 b**), WT cells appeared healthy but had not reached confluence, in contrast cells without FCS and treated with FGF18 (**Fig. 5.5 a**) were surviving poorly with high levels of cellular death and few viable cells remained. Cells growing in 1% and 10% FCS with or without growth factor supplementation (**Fig. 5.5 d,f**

and **c,e**) were all confluent, although cells growing in 1% FCS with FGF18 added (**e**) demonstrated a higher level of cell death.

When mutated cells were observed at both time points, it was apparent that they tolerated the absence of FCS poorly. At 24 hours without FGF18 (**Fig. 5.6 B,D,F**) approximately half of the visible cells were dead if no FCS was present (**Fig. 5.6 B**). When FGF18 was added, no viable cells were seen (**Fig. 5.6 A**). Cell survival was improved in the presence of 1% FCS (**Fig. 5.6 D,C**), however, the presence of FGF18, again conferred a high level of cell death (**Fig. 5.6 C**). With 10% FCS similar cell numbers were observed although cells treated with FGF18 (**Fig. 5.6 E**) appeared more stressed. At 72 hours only a few viable cells were seen in the cultures without FCS or growth factor (**Fig. 5.7 b**), when growth factor had been added only cellular debris was seen (**Fig. 5.7 a**). When 1% FCS was added (**Fig. 5.7 d**) cell survival was greatly enhanced. When FGF18 was also added (**Fig. 5.7 c**) a greater number of cells were observed, although a higher level of cellular stress was also noted. In the presence of 10% serum without FGF18 (**Fig. 5.7 f**) C278F cells took on their subconfluent appearance, whereas when treated with FGF18 (**Fig. 5.7,e**) they became fully confluent, although multiple dead cells were apparent.

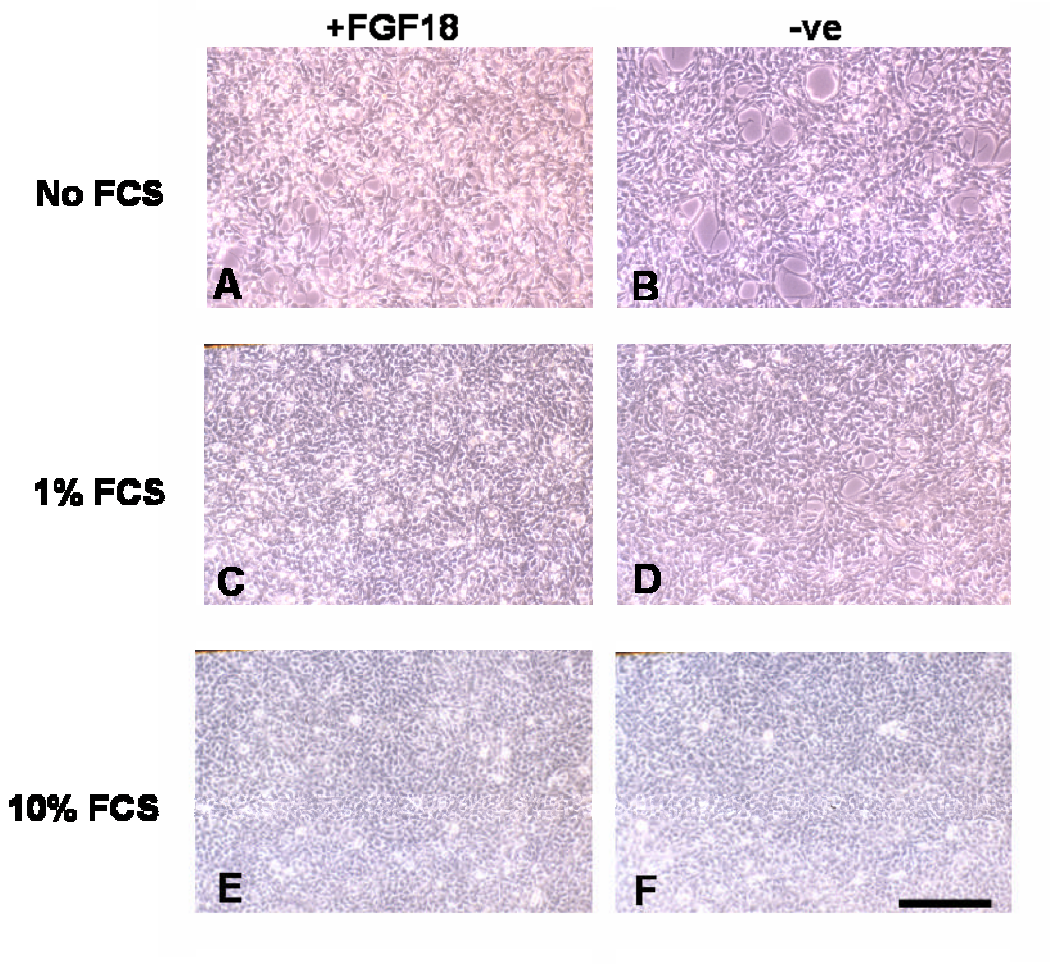


Figure 5.2: Effects of foetal calf serum and FGF18 on MC3T3-E1 cell growth at 24 h

Cells at 24 hours with FGF18[10^{-9}] (**A,C,E**) or without treatment (B,D F), without serum (**A,B**), with 1% (**C,D**) or 10% (**E,F**) serum.

Cells were seeded at 12,000/cm^2 in normal medium. Medium was replaced after 12 hours with the treatment medium. Cultures were imaged by phase contrast after 24 of treatment. Scale bar = 200 µm

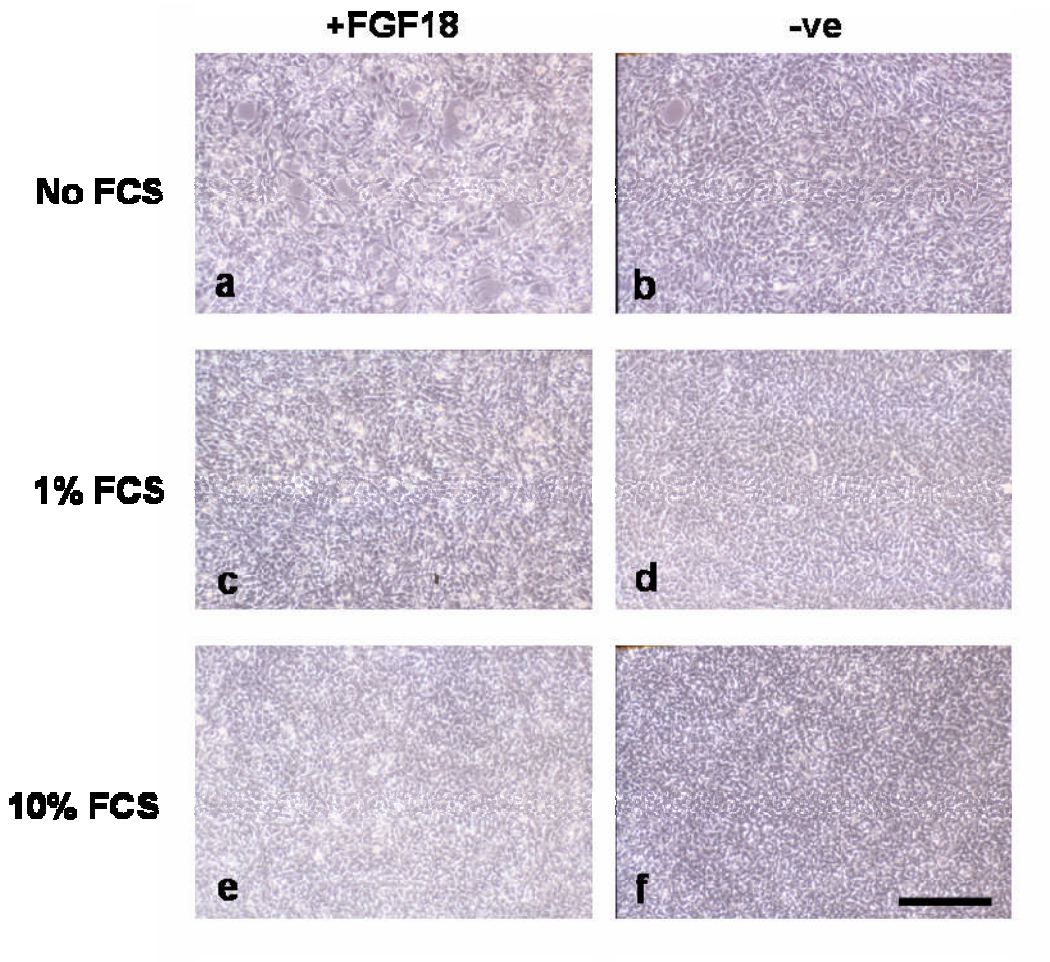


Figure 5.3: Effects of foetal calf serum and FGF18 on MC3T3-E1 cell growth at 72 h

Cells at 72 hours with FGF18[10^{-9}] (**a,c,e**) or without treatment (**b,d,f**), without serum (**a,b**), with 1% (**c,d**) or 10% (**e,f**) serum.

Cells were seeded at 12,000/cm^2 in normal medium. Medium was replaced after 12 hours with the treatment medium and every 24 hours thereafter. Cultures were imaged by phase contrast after 72 of treatment. Scale bar = 200 µm

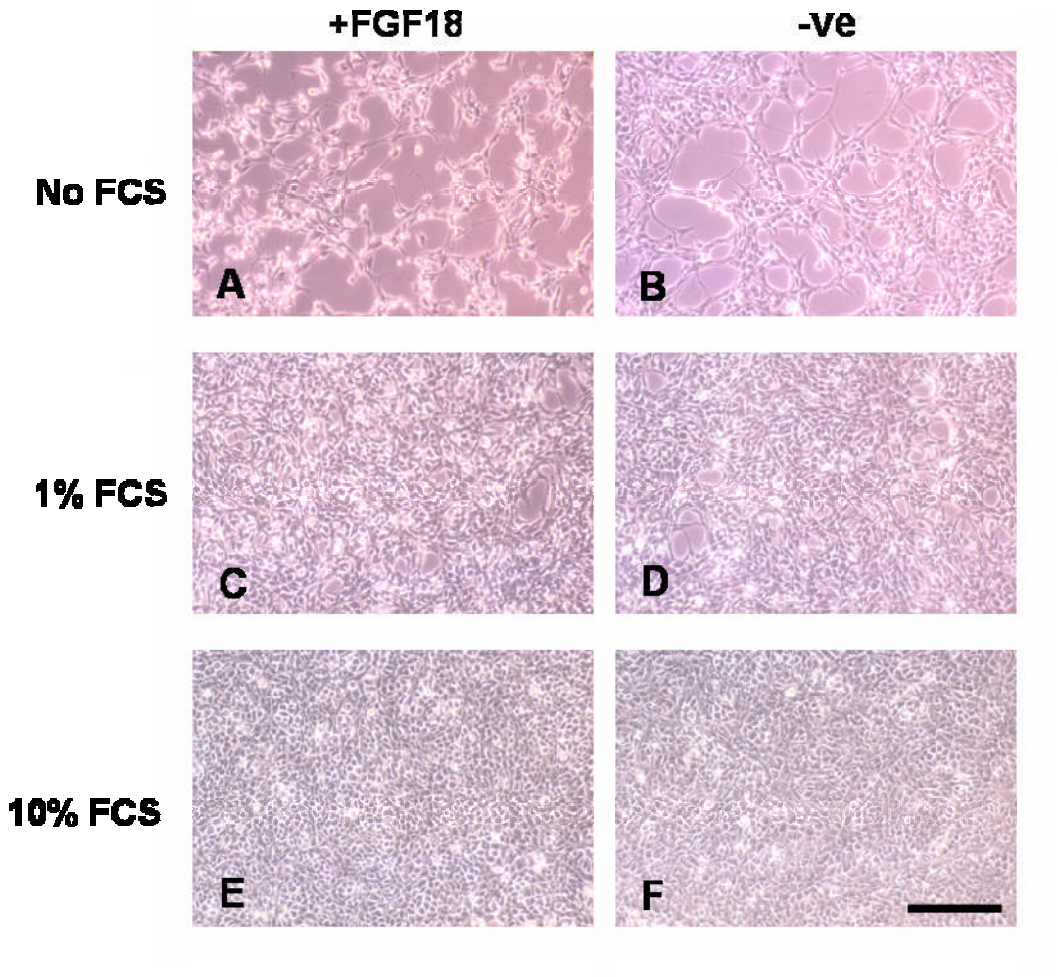


Figure 5.4: Effects of foetal calf serum and FGF18 on WT cell growth at 24 h

Cells at 24 hours with FGF18[10^{-9}] (**A,C,E**) or without treatment (B,D F), without serum (**A,B**), with 1% (**C,D**) or 10% (**E,F**) serum.

Cells were seeded at 12,000/cm^2 in normal medium. Medium was replaced after 12 hours with the treatment medium. Cultures were imaged by phase contrast after 24 of treatment. Scale bar = 200 µm

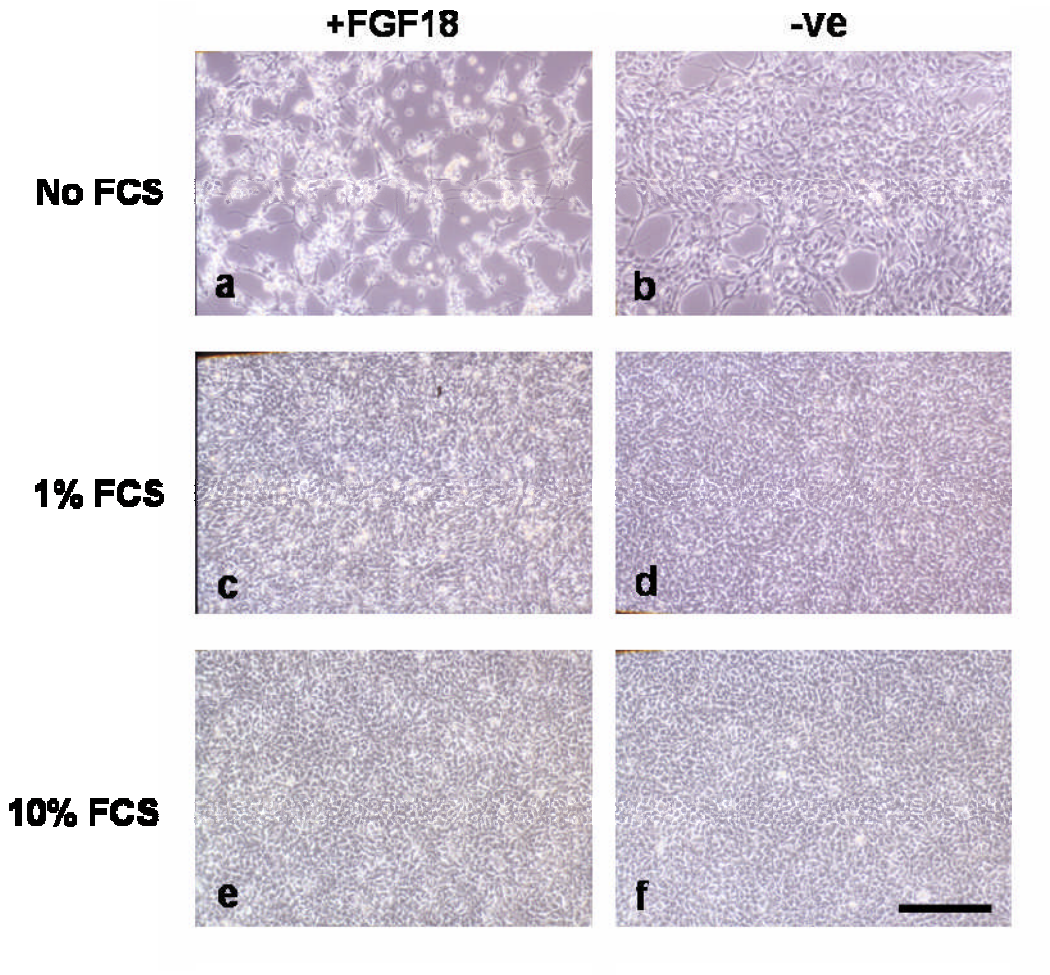


Figure 5.5: Effects of foetal calf serum and FGF18 on WT cell growth at 72 h

Cells at 72 hours with FGF18[10^{-9}] (**a,c,e**) or without treatment (**b,d,f**), without serum (**a,b**), with 1% (**c,d**) or 10% (**e,f**) serum.

Cells were seeded at 12,000/cm^2 in normal medium. Medium was replaced after 12 hours with the treatment medium and every 24 hours thereafter. Cultures were imaged by phase contrast after 72 of treatment. Scale bar = 200 µm

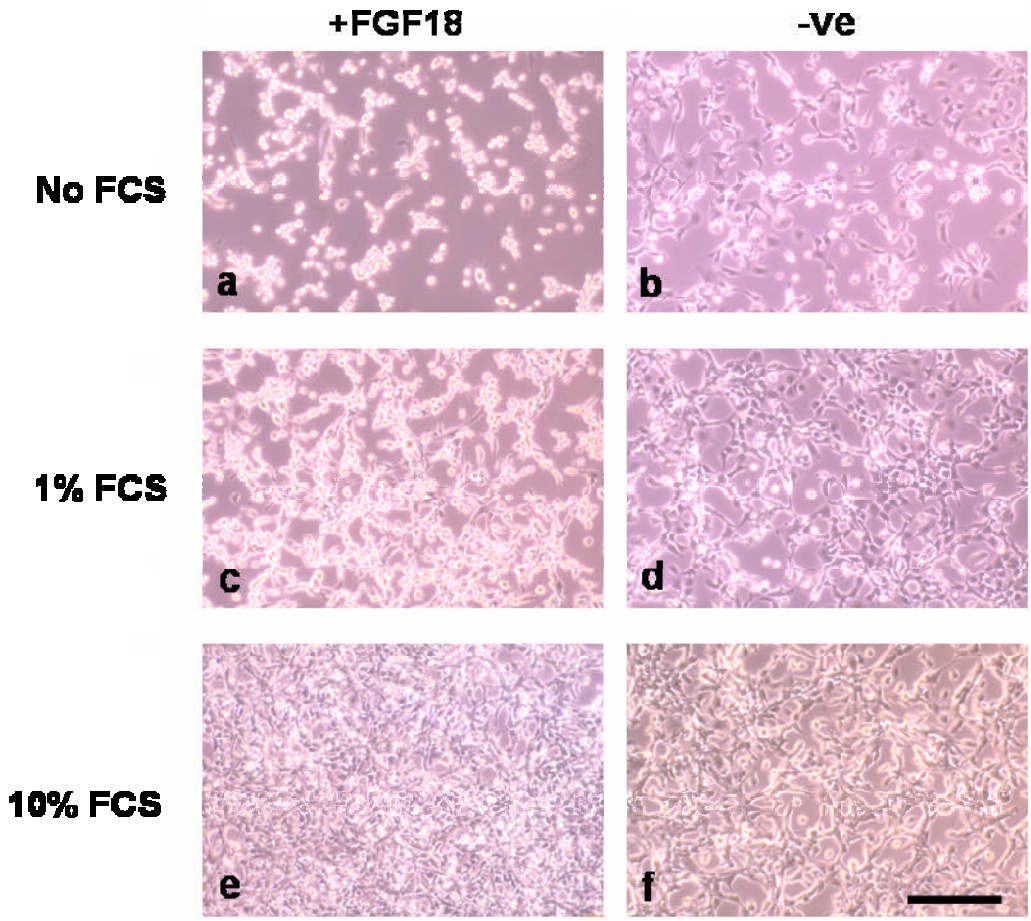


Figure 5.6: Effects of foetal calf serum and FGF18 on C278F cell growth at 24 h

Cells at 24 hours with FGF18[10^{-9}] (**A,C,E**) or without treatment (**B,D F**), without serum (**A,B**), with 1% (**C,D**) or 10% (**E,F**) serum.

Cells were seeded at 12,000/cm^2 in normal medium. Medium was replaced after 12 hours with the treatment medium. Cultures were imaged by phase contrast after 24 of treatment. Scale bar = 200 µm

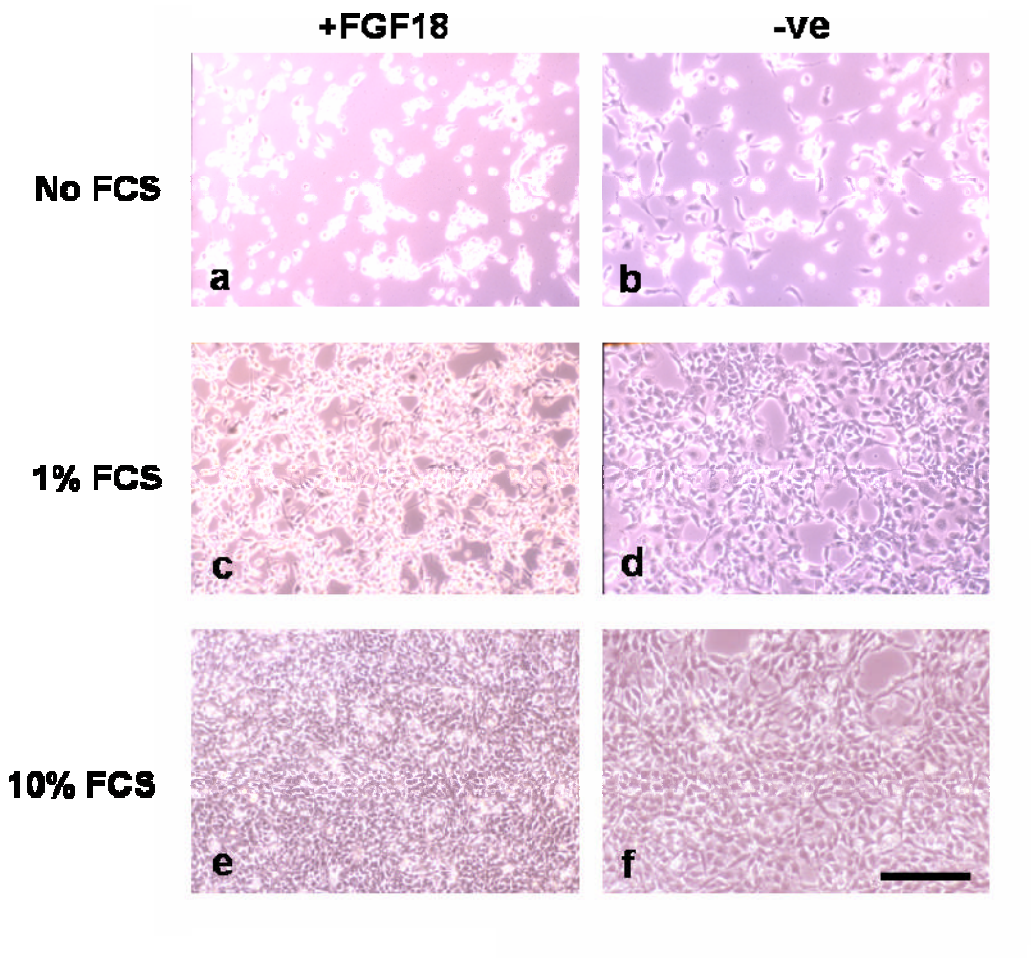


Figure 5.7: Effects of foetal calf serum and FGF18 on C278F cell growth at 72 h

Cells at 72 hours with FGF18[10^{-9}] (**a,c,e**) or without treatment (**b,d,f**), without serum (**a,b**), with 1% (**c,d**) or 10% (**e,f**) serum.

Cells were seeded at 12,000/cm^2 in normal medium. Medium was replaced after 12 hours with the treatment medium and every 24 hours thereafter. Cultures were imaged by phase contrast after 72 of treatment. Scale bar = 200 µm

5.3.3: Proliferation effects of FGF18 treatment

Treatment with FGF18 produced an obvious increase in cell numbers amongst all cell lines, but most strikingly in the mutated cells, allowing them to grow to full confluence which until this stage, had not been observed. This apparent proliferative enhancement was quantified using anti-phosphohistone3 (pH3) as described in chapter 4.

Observations from the previous section revealed that the mutated cells required FCS to survive. While the addition of 1 % FCS obviously improved the survival of mutated cells, they continued to show a high degree of cellular stress, particularly in the presence of growth factor. Therefore further observations of cellular growth with different concentrations of FCS in the presence of FGF18 were performed and it was elected to carry out the experiment with all cultures growing in 2% FCS. This concentration allowed all lines to grow, without showing excessive signs of cellular stress.

Treatment with FGF18 increased the proliferation of all cell lines. This effect was greatest in the first 24 hours of treatment and a visual impression of this is gained by comparison of day 1 and day 2 staining in **Figures 5.8** and **5.9.** This was confirmed by analysis of the mitotic indices, calculated for each line and time point as shown in **Figure 5.10.** Proliferation was significantly enhanced in a concentration dependent manner most notably for the WT and C278F lines. On day 1 this effect was greatest for mutated cells, producing a 2.4 fold increase in proliferation at [10^{-8}M] compared to a 1.9 fold increase for both MC3T3-E1 and WT cells. At day 2 the effect was less obvious and there was no statistically significant difference in the increase between lines.

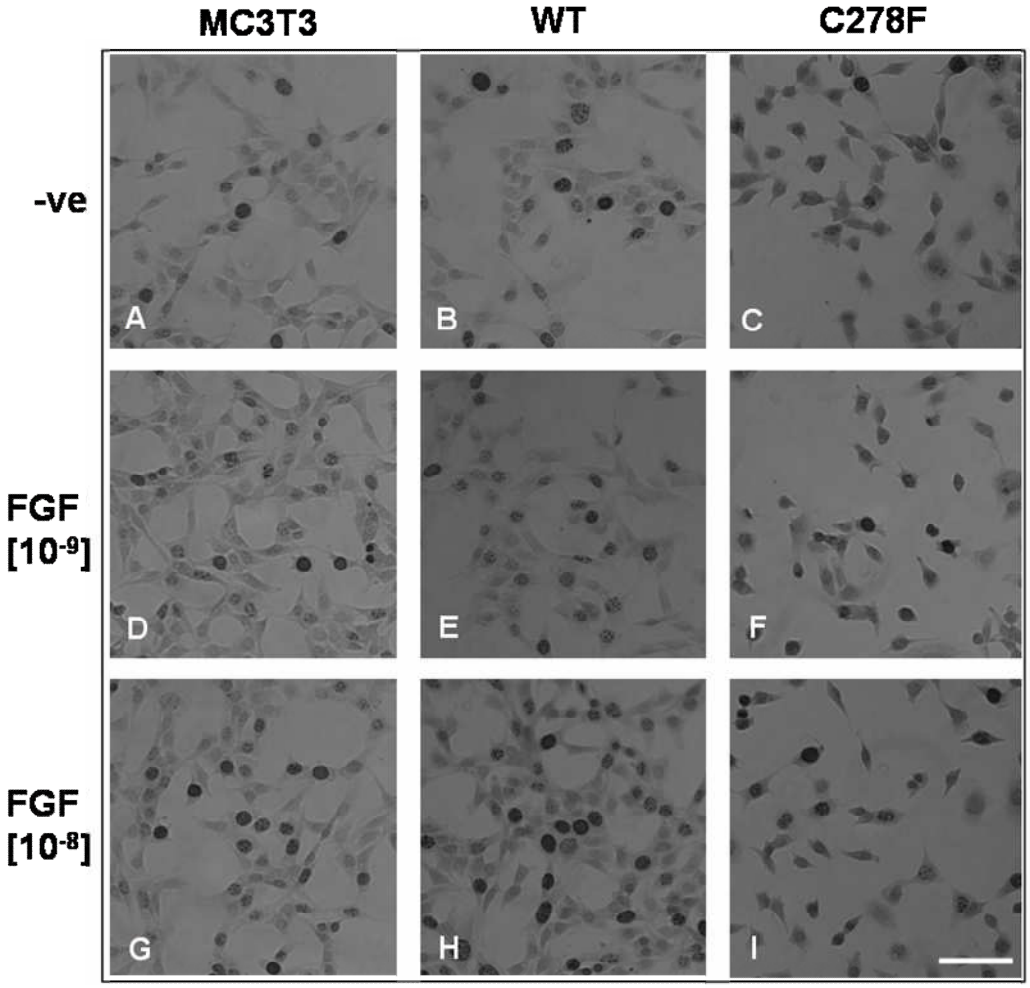


Figure 5.8: Effect of FGF18 on proliferation using anti-pH3. Day 1

Cells were seeded in triplicate at 5000/cm^2 in normal medium. Medium was replaced after 12 hours with treatment medium containing 2% FCS in the absence (**A-C**) of FGF18 or supplemented with FGF18[10^{-9} M] (**D-F**) or FGF18[10^{-8} M] (**G-I**). Cultures were immunostained for anti-pH3 after 24 hours of treatment and imaged by phase contrast for measurement of mitotic index using Openlab software "click counting" facility. (gain/contrast adjusted images not shown). Scale bar = 50 µm

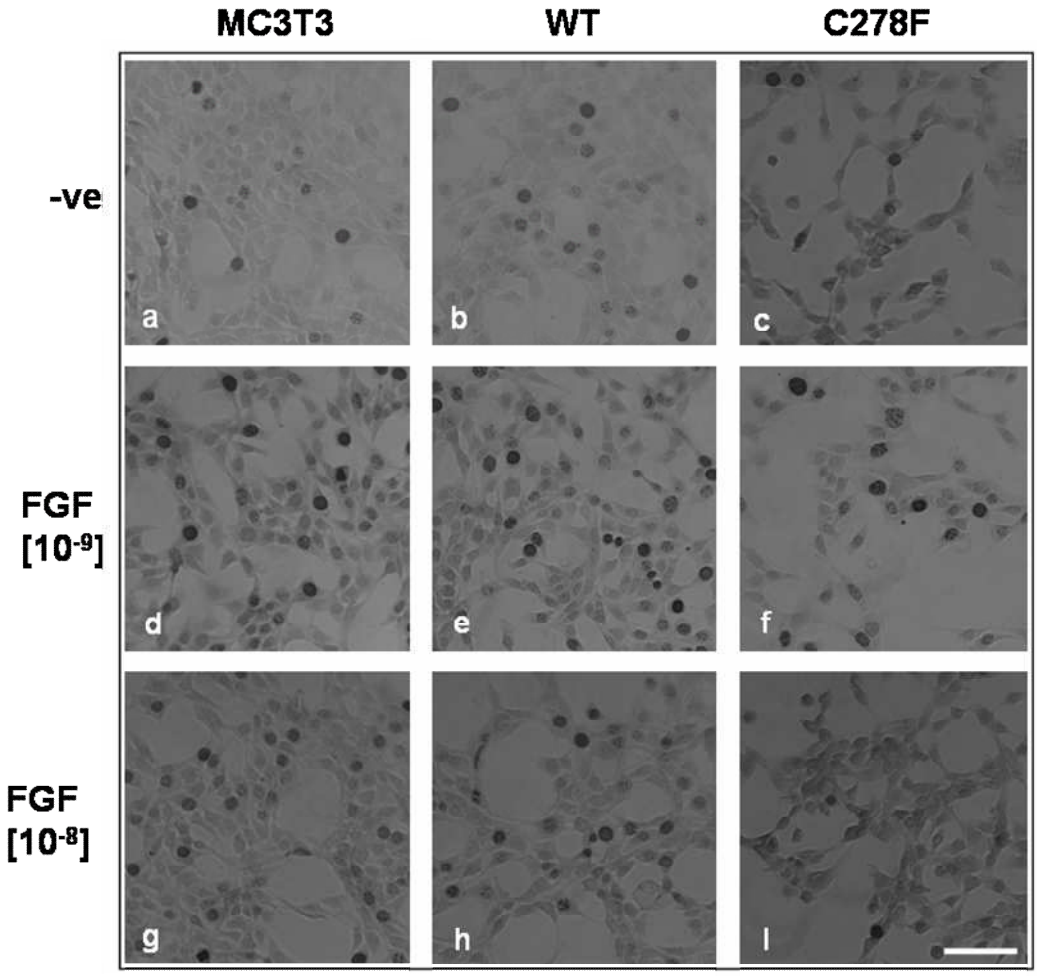


Figure 5.9: Effect of FGF18 on proliferation using anti-pH3. Day 2

Cells were seeded in triplicate at 5000/cm^2 in normal medium. Medium was replaced after 12 hours with treatment medium containing 2% FCS in the absence (**a-c**) of FGF18 or supplemented with FGF18[10^{-9} M] (**d-f**) or FGF18[10^{-8} M] (**g-i**) which was changed after 24 hours. Cultures were immunostained for anti-pH3 after 48 hours of treatment and imaged by phase contrast as before. Scale bar = 50 µm

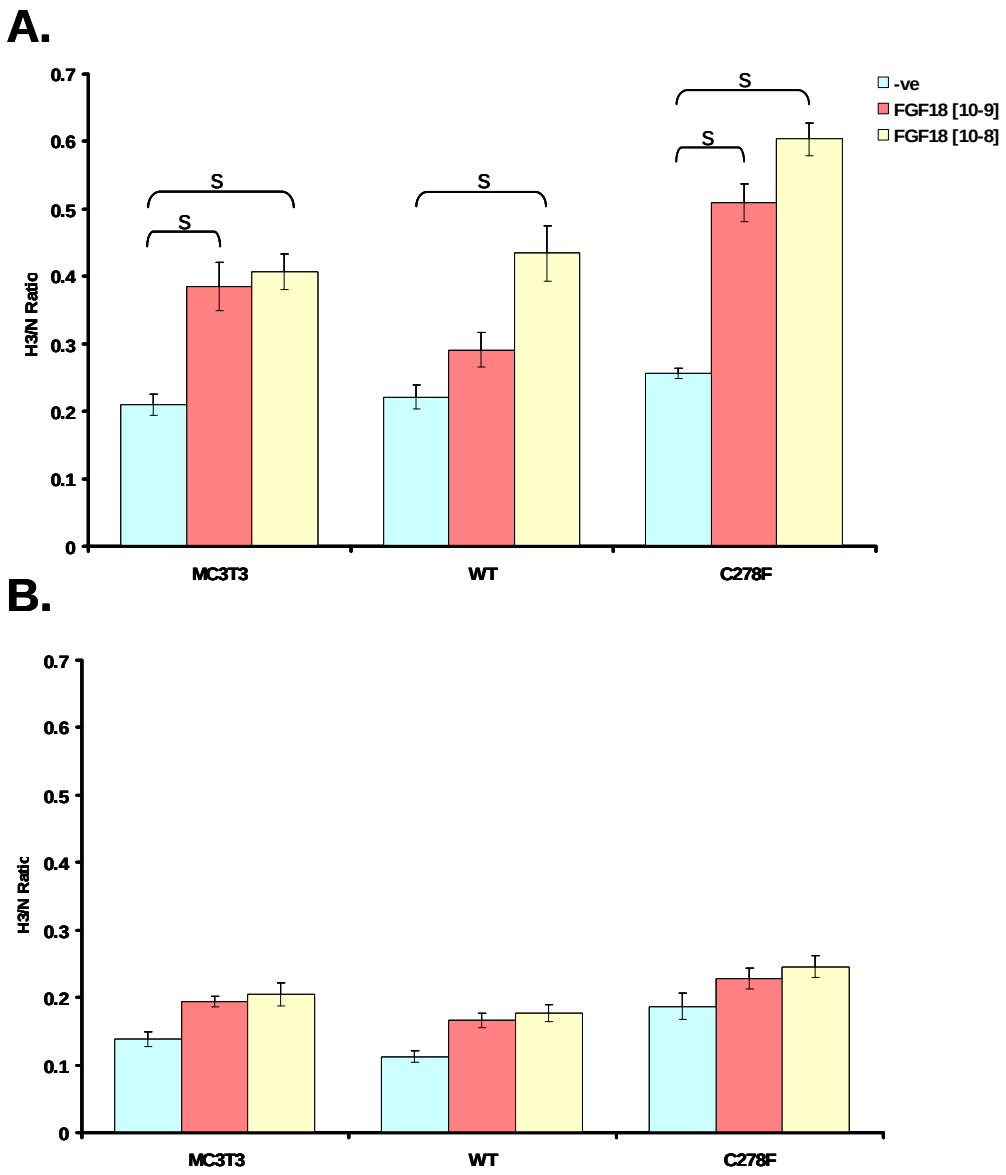


Figure 5.10: Effect of FGF18 on mitotic index.

Graphic representation of mitotic index, assessed by anti-pH3 staining

A. Day 1. Ratio of anti-pH3 positive cells as proportion of total cell count for MC3T3-E1, WT and C278F cells.

B. Day 2.

Data are expressed as the mean ± SEM for each group (n=12). Statistical differences among treatment groups were evaluated using analysis of variance (ANOVA) and the significance of differences between groups was determined by *post hoc* testing using Bonferroni's method. **S** = significant ($p<0.05$ was considered significant)

5.4: Discussion

5.4.1: Effect of FCS

During initial observations of cell culture in chapter 4, it was observed that the mutated cells seemed more dependent on serum supplementation than did the MC3T3-E1 or WT cells. This was further emphasised whilst optimising culture conditions before quantifying the effects of FGF18 treatment. MC3T3-E1 cells tolerated serum starvation well as did WT cells in the absence of FGF18, whereas C278F cells did not tolerate serum starvation. The addition of FGF18 in the absence of serum had a severely deleterious effect on WT and C278F cell survival, in contrast to reports by Hill et al., (1998) and Debiais et al., (2004), that FGF2 rescues serum starved mouse primary calvarial osteoblasts and human primary osteoblasts respectively.

The addition of 1% FCS rescued the behaviour of WT cells. However 2% FCS was required in order to normalise the appearance of C278F cells. It became apparent that in conditions of low or absent FCS, FGF18 treatment was causing cell death, whereas the addition of FGF18 at higher concentrations of FCS, enhanced cell numbers. This was seen most obviously during the preliminary finding that C278F cells reached full confluence in the presence of 10% FCS and FGF 18 [10^{-9}M]. This had not previously been observed in the C278F cells. In addition there was no evidence that the cells were forming nodules early. The effect also appeared to be dependent on FGF18 concentration, as the addition of FGF at [10^{-10}M] modified the appearance of C278F cells towards confluence.

Taken together, these observations suggested that FGF18 acting alone was driving cell death, but that in the presence of other growth factors and serum components, was enhancing cell survival either by increasing proliferation, reducing apoptosis or a combination of both. In addition, the absence of clustering and early nodule formation suggested that cell function was being affected. These observations might be explained in part and would be consistent with the tensegrity model. Such that low serum concentrations provided suboptimal levels of absorbed extracellular matrix proteins including fibronectin for example, for integrin associated attachment. The mutated cells therefore attach poorly, remain round and undergo apoptosis.

5.4.2: Effects of FGF18

Quantification of the proliferative effect of FGF18 in 2% FCS, revealed a concentration dependent increase in cellular mitosis for all cell lines. This was significantly greater at day 1 for the mutated cells than for the MC3T3-E1 and WT cells. However, the overall magnitude of increase was not seen to the same degree as Shimoaka et al., (2002), who carried out treatment in 10%FCS. This suggests that other growth factors present in the serum work in synergy with the FGF18 to influence proliferation. Furthermore, when cells were serum starved or at very low concentrations of FCS, the degree of cell death, possibly apoptotic in nature, was visually much greater in the mutated cells. It can be postulated that this response of the C278F cells suggests that the lack of cellular attachment and spreading via the ECM, in combination with a substance which under different conditions is mitogenic, is causing cellular death. This observation is consistent with both the more rounded appearance of the mutated cells in this study as predicted by the tensegrity model and the findings by Mansukhani et al., (2000), who observed that more mature cells respond to FGF treatment by undergoing apoptosis.

The observed concentration effect on proliferation differs from previous findings for FGFs, particularly FGF2, where it has been found that at low concentrations, FGF2 promotes proliferation and at higher concentration proliferative arrest and differentiation is observed (Iseki et al., 1999; Moore et al., 2001; Sarkar et al., 2001). This study was unable to examine the effect of concentrations higher than 10^{-8} Molar. Had this been possible it would have been interesting to assess whether cells switched from proliferation to differentiation upon treatment with FGF18 at higher concentrations.

The mutation carried by the C278F cells causes ligand independent constitutive activation of the receptor (Robertson et al., 1998) and should therefore not respond to exogenous FGF. Moreover, it is possible that the constitutively activated FGFR2 creates a negative feedback, causing down-regulation of the receptor, a theory which has subsequently been supported by the recent findings of Hatch et al., (2006), as discussed in Chapter 4. It is therefore likely that the early increase in proliferation by FGF18 in the mutated cells is caused by the activation of other endogenous wild type FGF receptors, which may have differentially up-regulated expression secondary to the autocrine influence of the mutated FGFR2 receptor. It would therefore have been informative to look at the expression profiles of the FGFRs following FGF18 treatment.

In addition to increased proliferation, the full confluence of mutated cells without the tendency to form nodules prematurely was observed and it is tempting to speculate that treatment with FGF18 is affecting cell-cell interaction and adhesion. Further studies

examining the expression of some of the likely candidates should be considered in future work.

5.4.3: Summary

In this chapter, it was observed that at low serum concentrations or in the absence of serum, FGF18 treatment was associated with cell death and that this was seen to the greatest degree in cells carrying the C278F mutation. When serum concentration was increased to a level which supported normal cell survival, treatment with FGF18 increased proliferation and this effect was greatest in pre-confluent cultures of C278F cells. In addition, treatment with FGF18 at concentrations at or above [10^{-10}M] "normalised" the appearance of the C278F cultures by allowing the cells to become fully confluent without early clustering. The effect of FGF18 treatment on the expression of osteodifferentiation and cellular attachment proteins would have been a natural further extension of this study.

Having observed that behaviour of the mutated cells could be influenced by modification of the culture environment, in a manner which seemed beneficial to the expansion of the cellular population, it was also necessary as part of an integrated approach, to look for substances which might act as an appropriate scaffold, on which to transfer osteoprogenitors to surgical sites requiring bony regeneration.

Chapter 6: Bioabsorbable Membranes & Substrate Attachment

6.1: Introduction

The development and widespread introduction of internal metallic bone fixation over the past 25 years has been one of the most significant advances in craniomaxillofacial surgery since the basic concepts of secure bone fixation and primary bone healing were introduced several decades previously. With the extensive use of fixation technology in paediatric craniofacial surgery in particular, it is now apparent that there are several potential postoperative complications that may occur as discussed in chapter 1. Given the young age at which the paediatric craniofacial procedures are performed, some patients will require device removal in their lifetime (Rubin & Yaremchuck, 1997). More significantly, the development and bone apposition/resorptive pattern of skull growth, creates the potential for growth restriction and eventual translocation of metal fixation devices to the endocranial surface (Munro et al., 1993). The tips of screws against the dura or juxtaposed to the cerebral cortex impose a risk of creating an injury to the brain surface and seizure focus, (Fearon et al., 1995). The use therefore, of resorbable devices in an attempt to prevent the associated complications, has been the subject of much study and clinical evaluation.

The first published clinical use of resorbable devices was in 1972 by Cutright and Hunsuck, who used non load-bearing polylactide sheets for orbital floor repair. Subsequently, more complex patterns including screws and fixation plates were introduced into clinical practice in the late 1970s and early 1980s. (Sedel et al., 1978; Rokkanen et al., 1985). Resorbable devices are now commonly used for procedures requiring bone fixation and having been used successfully and safely in this context, there has been a natural endeavour to extend their application to one of tissue engineering.

Whilst many workers have studied the interaction of osteoprogenitor cells on numerous potential scaffolds, there remains a scarcity of work investigating the interaction of these scaffolds with osteoprogenitors carrying mutations affecting osteogenisis. It was therefore important to firstly discover whether osteoprogenitors carrying the FGFR2-C278F

mutation would be attracted to and attach to bioresorbable scaffolds which might be candidates for a bone engineering application.

In the absence of a novel material which had been pledged for study, a commercially available co-polymer plate (*BioSorbPDX*) which was already being widely used for bone fixation in the clinical setting was chosen.

Having also observed (Chapter 4) that cells carrying the C278F mutation did not attach to culture plastic so readily and detached more easily than the wild type and untransfected cells, it was envisaged that it maybe possible to optimise the attachment, growth and differentiation of these cells on scaffolds by surface modification with the use of coating agents. For this purpose, the matrix proteins fibronectin and laminin were chosen having previously been studied and shown to affect attachment of several cell types to different materials (Cargill et al., 1999; Rezania & Healy, 1999; Yang et al., 2001; Stephansson et al., 2002).

In order to combine the attributes of the PLGA co-polymer and bioactive glasses which have been shown to be osteoconductive or even osteoinductive (Yuan et al., 2001) but which have poor tensile strength and are brittle, a novel composite scaffold made of bioactive glass fibres and the biodegradable PLGA co-polymer was also assessed for its ability to attach and sustain osteoblasts.

6.2: Results

6.2.1: Early attachment to untreated co-polymer plates

Initial attempts to grow the osteoblast cell lines on the *BioSorbPDX* plate **(Figure 6.1**) revealed that cells were slow to attach compared to tissue culture wells. Whereas MC3T3-E1 cells showed no sign of attachment to *BioSorbPDX* plates (**A**), cells were well attached on culture plastic at 24 hours post plating (**B**). However, cells eventually attached and at 4 days post plating, several cells could be seen with pre-confluent morphology on the *BioSorbPDX* surface (**C**). In contrast cells grown on culture plastic had a typical confluent or near-confluent morphology with visibly greater numbers (**D**).

The poor early attachment of cells to co-polymer plates led to the seeding of cells at significantly higher densities for subsequent experiments involving the plates and coating agents. This took into consideration the ultimate target of achieving efficient cellular expansion upon the scaffolds prior to normal differentiation and in addition allowed for the

finite availability of the plates and coating agents. There was also recognition of the time constraints upon the study. For similar reasons, experiments were subsequently confined to the WT and C278F cell lines.

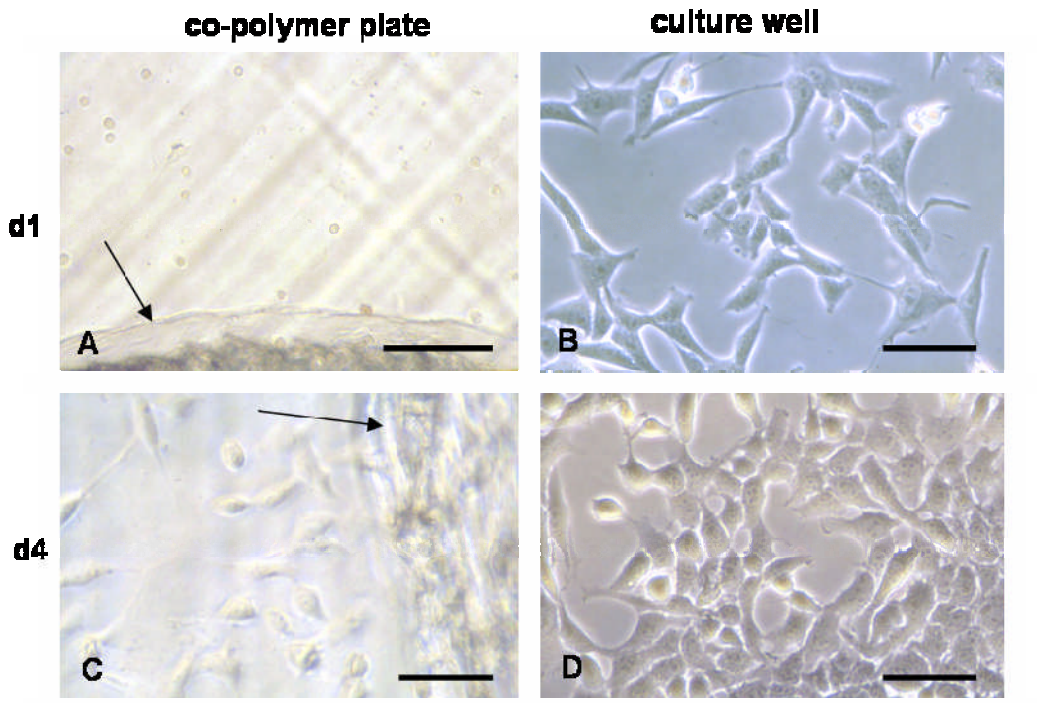


Fig 6.1: MC3T3-E1 cells growth on co-polymer plate (*BioSorbPDX)* and culture well
Non-attached cells on copolymer plate at 24h (**A**). Cells attached to culture plastic at 24h (**B**). Cells attached to copolymer plate at 4d (**C**). Near confluent cells on culture plastic at 4d (**D**). The parallel line pattern seen in (**A**) represents the surface profile markings created during manufacture of the plates. Arrows represent the edge of the bevelled screw hole. Cells seeded at 20,000/cm^2. Images obtained by phase contrast microscopy.
Scale bars = 200 µm in (**A**); 50 µm in (**B-D**)

6.2.2: Effect of laminin and fibronectin treatment on cellular attachment

Before attempting to enhance attachment of cells to the *BioSorbPDX* plates, the effect of the two commercially available substrates, laminin and fibronectin on wild type and mutated osteoblast growth on tissue culture plastic was quantified. High magnification, phase contrast images of mutated cells (**Figure 6.2**) demonstrated that after two hours there is was observable enhancement of cellular attachment, manifest by more highly developed filopodia and visible nucleoli in wells coated with fibronectin and laminin (not shown).

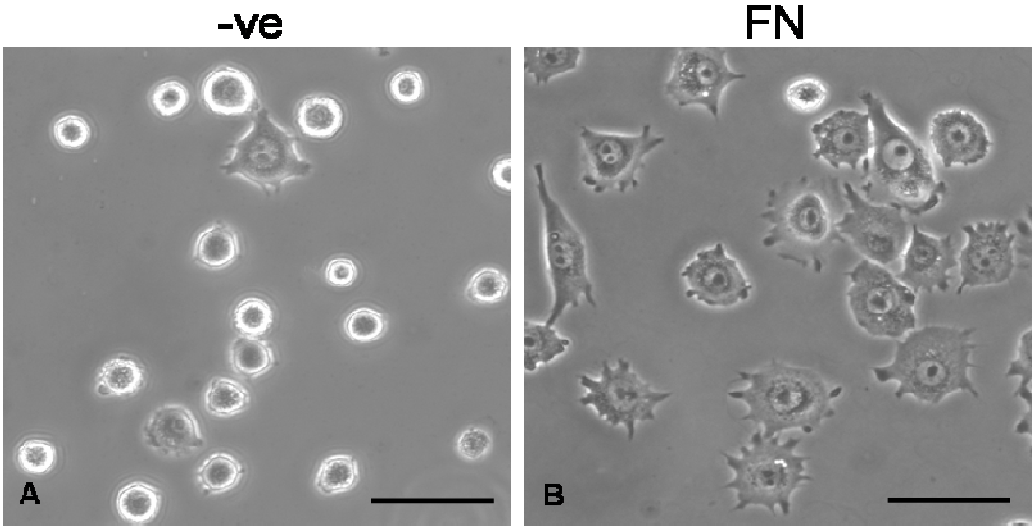


Figure 6.2: The effect of fibronectin on early attachment of C278F cells
Cells grown on untreated culture wells two hours after seeding (**A**), and cells on fibronectin coated wells at two hours (**B**). Cells were seeded at 30,000cells/cm^2. Images obtained by phase contrast microscopy. Scale bars = 25 µm

Wild type and C278F cells were seeded on 12-well plates coated with fibronectin or laminin or on untreated wells (-ve control). Wells were imaged at 1, 4 & 24 hours (**Fig. 6.3 and 6.4**) and triplicated samples for each condition and time point were released for counting at 1, 2, 4 and 48 hours post plating (**Fig. 6.5**). Approximately half of the WT cells appeared to show early signs of attachment one hour after seeding in unprepared culture wells (**Fig 6.3 A**), whereas virtually all cells seeded on wells coated with fibronectin and laminin had attached (**Fig 6.3 B,C**). After 4 hours in culture, most cells in unprepared

wells demonstrated signs of early attachment (**Fig 6.3 D**), compared to the visible attachment of all viable cells in the fibronectin and laminin coated wells (**Fig 6.3 E,F**). After 1 day in culture without coating, all viable cells had attached (**Fig 6.3 G**). However, cells growing on fibronectin (**Fig 6.3 H**) had undergone considerable proliferative expansion and those grown on laminin were near confluent (**Fig 6.3 I**). Two days after plating (not shown) cells on fibronectin and laminin were fully confluent whereas negative controls were approximately 70-80% confluent.

Mutated cells (**Fig. 6.4**) one hour after seeding in uncoated wells, demonstrated minimal signs of attachment (**Fig 6.4 A**). Approximately half of the cells seeded on wells coated with fibronectin (**Fig 6.4 B**) were attached and nearly all cells on laminin (**Fig 6.4 C**) showed signs of attachment. After 4 hours in culture there were progressive signs of attachment, with fibronectin and laminin coated wells (**Fig 6.4 E,F**) showing the more typical morphology of well attached osteoblasts. After 1 day in culture without coating, most cells had attached and undergone a limited degree of expansion (**Fig 6.4 G**). Cells growing on both fibronectin and laminin were near confluent (**Fig 6.4 H,I**).

Quantification of cell numbers as described previously, are represented graphically in figure 6.5. Attachment of WT cells was significantly enhanced by both laminin and fibronectin at all time points although this effect was greatest for early cultures. Laminin and fibronectin had an equal effect on cell numbers in cultures of four hours or less. In later cultures at 48 hours, laminin had a statistically significant advantage on wild type cells compared to fibronectin which also significantly increased cellular attachment. Laminin and fibronectin significantly increased C278F cell numbers for time points up to 4 hours. There was no significant difference between laminin and fibronectin at 1 hour. However laminin had a significantly greater effect than fibronectin at 2 and 4 hours. Both substrates significantly increased cell numbers at 48 hours, although interestingly, the increase at this time point was significantly greater with fibronectin.

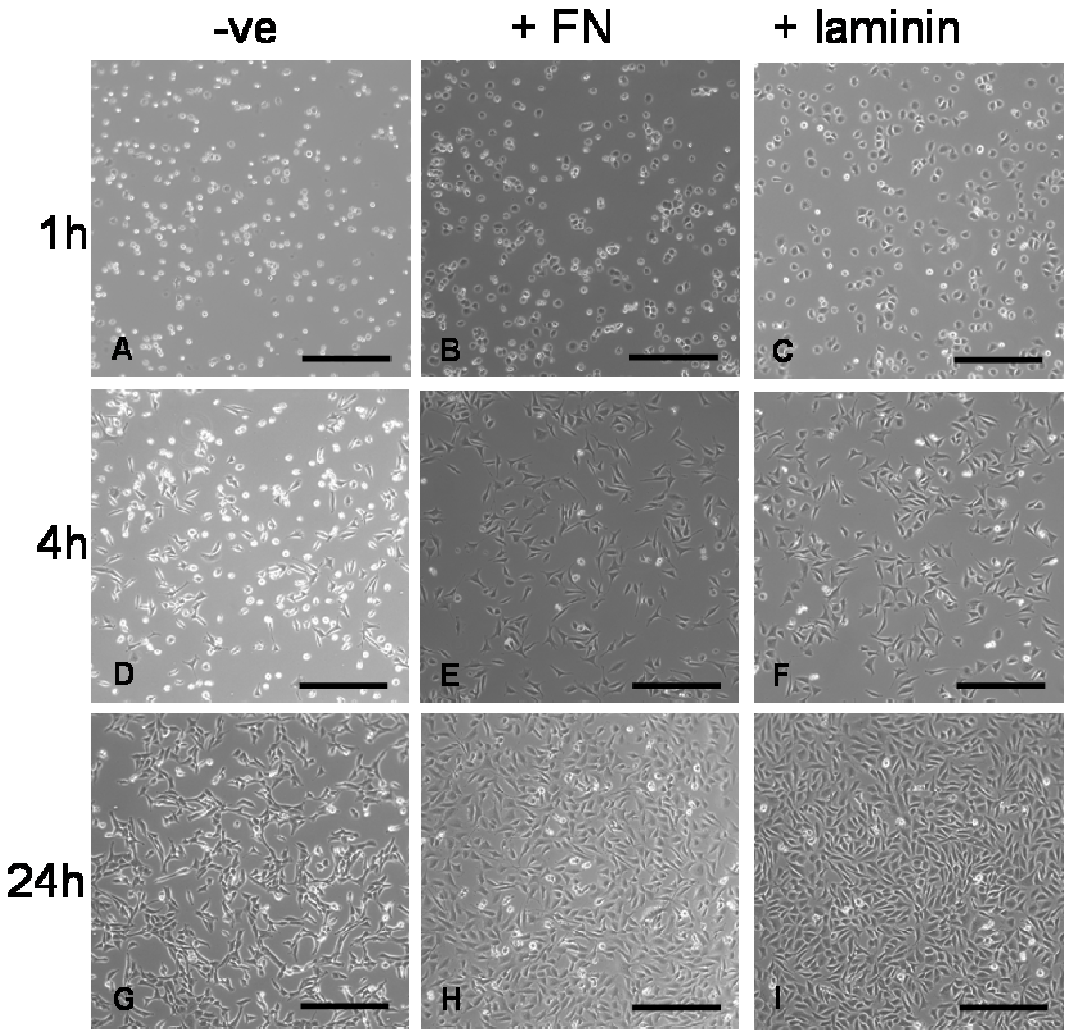


Figure 6.3: Effect of fibronectin & laminin coating on WT adhesion

Wild type cells were imaged at 1 hour (**A-C**), 4 (**D-F**) or 24 hours(**G-I**) after seeding in untreated wells(**A,D,G**), or in wells coated with fibronectin (FN) (**B,E,H**) or laminin (**C,F,I**). Approximately half the cells were attached at 1 hour on uncoated wells (**A**) compared to almost all cells on coated wells (**B,C**). After 4 hours, most cells showed early attachment on uncoated wells (**D**), whereas all viable cells were well attached on coated wells (**E,F**). At one day, all viable cells on uncoated wells had attached and appeared to have undergone some expansion (**G**), whereas cells grown on FN had undergone greater expansion (**H**) and cells grown on laminin were confluent (**I**). Cells were seeded at 30,000cells/cm^2. Images obtained by phase contrast microscopy. Scale bars = 200 µm

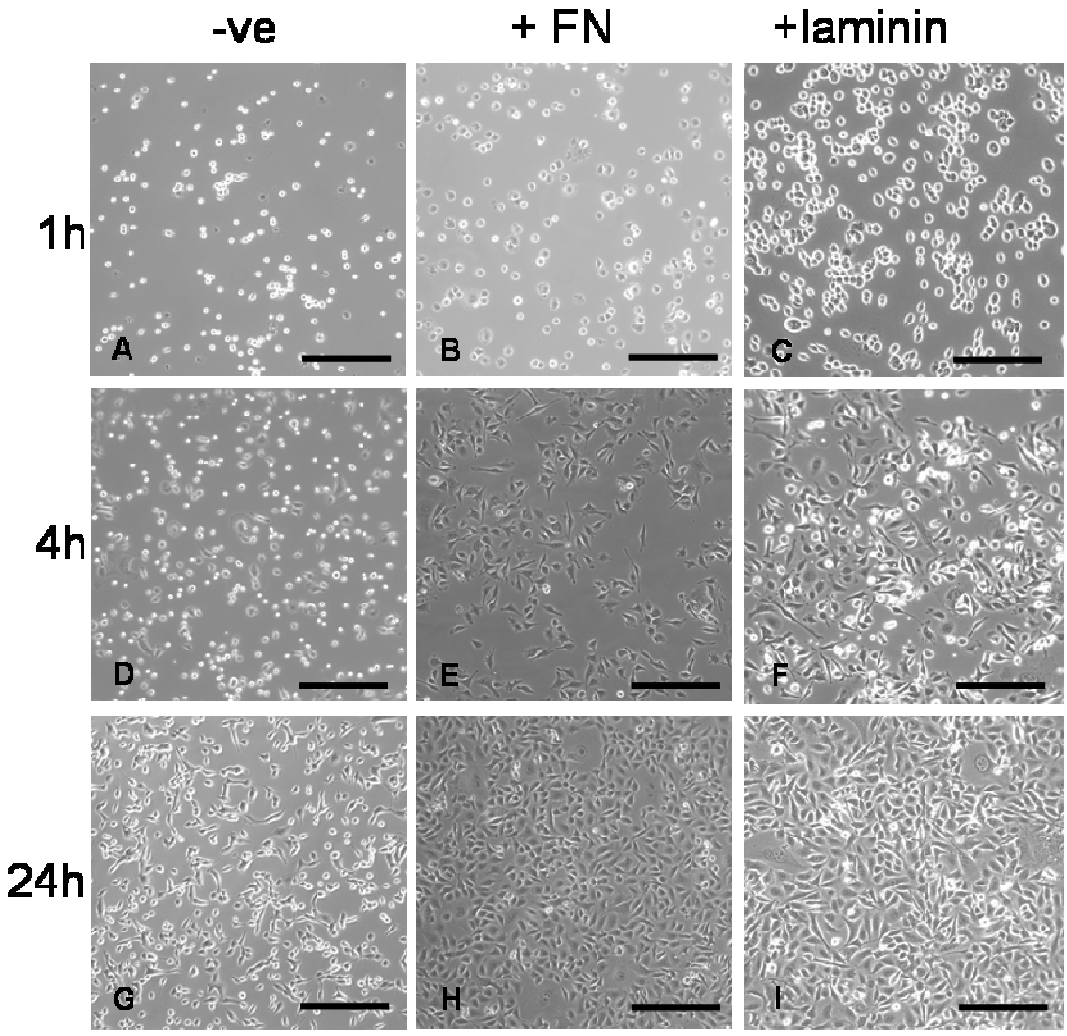


Figure 6.4: Effect of fibronectin & laminin coating on C278F adhesion

Cells carrying the C278F mutation were imaged at 1 hour (**A-C**), 4 (**D-F**) or 24 hours(**G-I**) after seeding in untreated wells (**A,D,G**), or in wells coated with fibronectin (FN) (**B,E,H**) or laminin (**C,F,I**). After 1 hour, cells grown on uncoated wells showed minimal attachment (**A**). Approximately half of the cells grown on FN (**B**) showed attachment and nearly all cells seeded on laminin (**C**) appeared to be attached. After 4 hours in culture cells in FN (**E**) and laminin (**F**) coated wells appeared well attached compared to non coated wells (**D**). After 1 day in the uncoated wells (**G**), cells were mostly attached, whereas cells grown on FN and laminin were near confluent (**H,I**) Cells were seeded at 30,000cells/cm^2. Images obtained by phase contrast microscopy. Scale bars = 200 µm

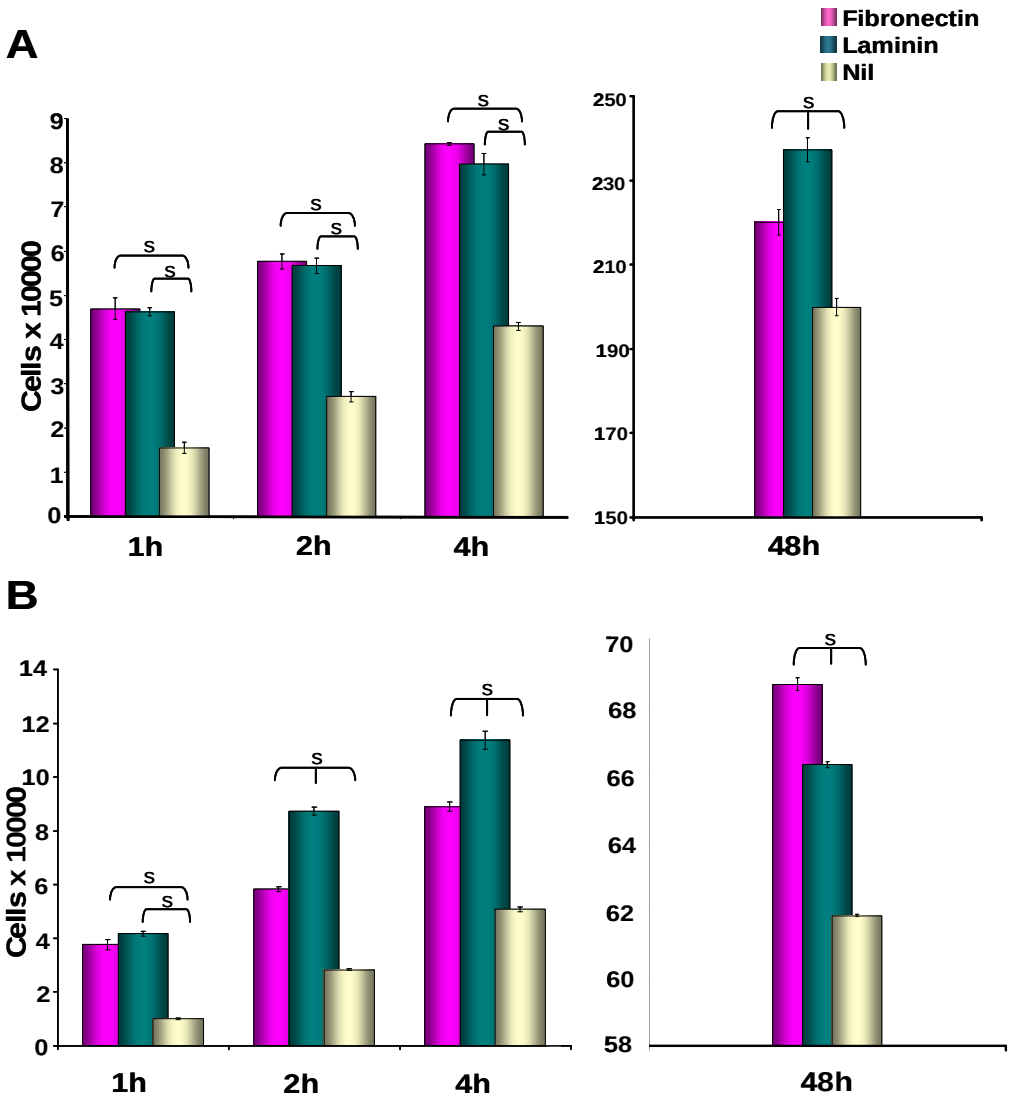


Figure 6.5: Effect of fibronectin & laminin coating on adhesion and growth of WTand C278F cells

Analysis of effect of fibronectin and laminin on wild type (**A**) and C278F (**B**) cells.

Cells were seeded as triplicates at 30,000/cm^2 in standard medium. Data are expressed as the mean ± SEM. Statistical difference amongst treatment groups (n=3) were evaluated using analysis of variance (ANOVA). Significance was determined by post hoc testing using Bonferronis's method. **S**=significant ($p < 0.05$ was taken as significant)

6.2.3: Effect of laminin and fibronectin treatment on cellular attachment to *BioSorbPDX* plates

Having shown significant attachment enhancement of both wild type and mutated cells to tissue culture plastic with laminin and fibronectin, the next step, having also observed the poor early attachment of osteoblasts to the *BioSorbPDX* plate, was to assess the potential of these two substrates to optimise the attachment, growth and mineralisation on the plates.

Observation of the *BioSorbPDX* plates within the first 1-6 hours (not shown), after seeding with C278F cells, revealed that early attachment had been enhanced by both fibronectin and laminin in a similar fashion to that observed when cells were seeded on treated culture dishes as shown in figure 6.4. Five days after seeding, cells were fully confluent on the fibronectin (**Fig. 6.6 C**) and laminin (**Fig. 6.6 E**) coated membranes, while some non-confluent areas remained on the untreated plate (**Fig. 6.6 A**). After 20 days of culture, multilayered nodules had developed in all conditions (**Fig. 6.6 B,D,F**) with nodules on the fibronectin coated plates appearing more developed (**Fig. 6.6 D**).

When compared with non coated plates, both substrates produced a significant increase in cell numbers at days 1 and 5 and this effect was significantly greater for laminin than fibronectin at both time points (**Fig. 6.7**). Surprisingly, at day 20, *BioSorbPDX* plates which had not been treated showed significantly greater cell numbers than either of the treatment groups. Of the two coating agents, fibronectin appeared to produce the greatest increase in cell numbers although this did not reach statistical significance.

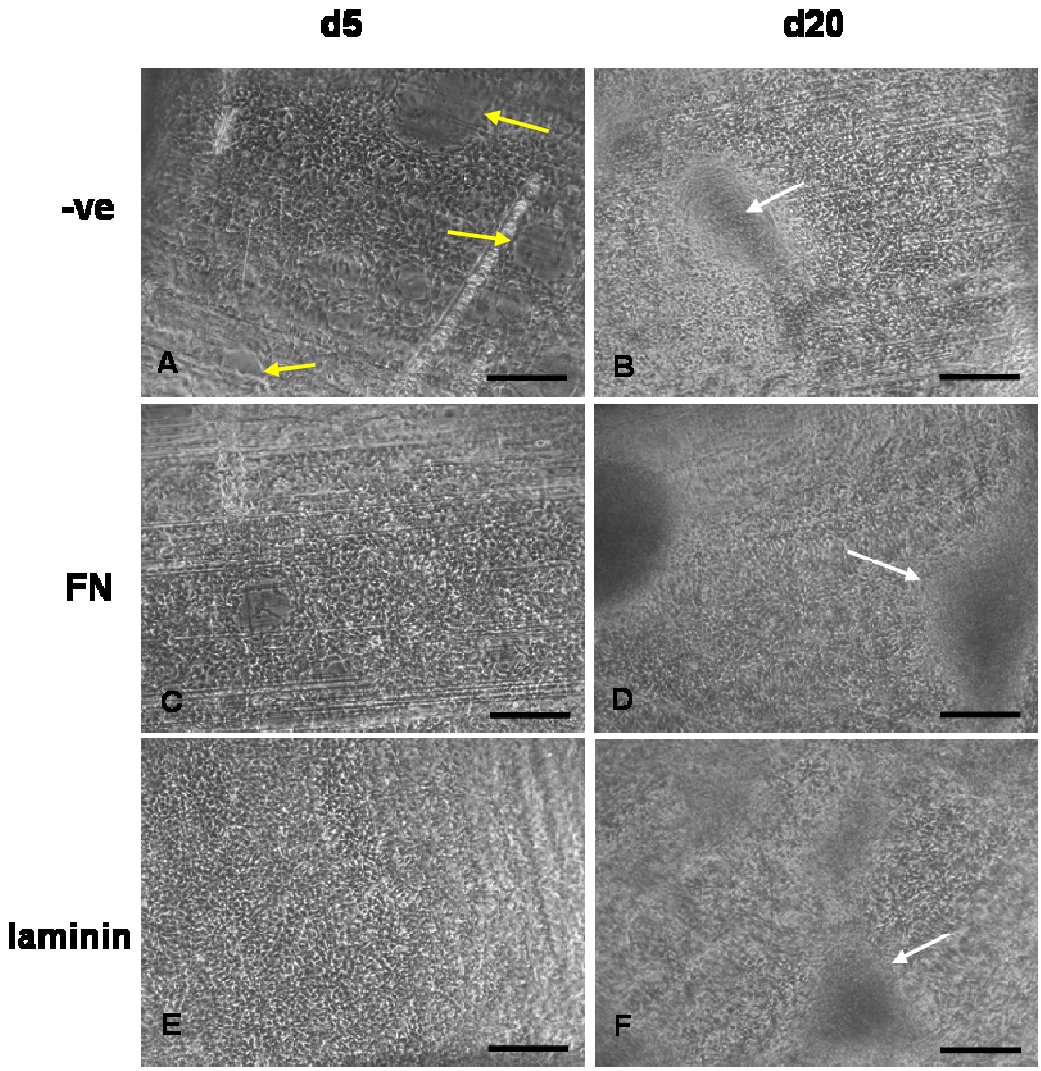


Figure 6.6: C278F cells on *BioSorbPDX* plate with fibronectin or laminin coatings. Five days after seeding, cells were fully confluent on the fibronectin (**C**) and laminin (**E**) coated plates. Non-confluent areas remained on the untreated plate (yellow arrows) (**A**). After 20 days of culture, multilayered nodules had developed in all conditions (**B,D,F**), as indicated by the white arrows. Scale bars = 200 µm

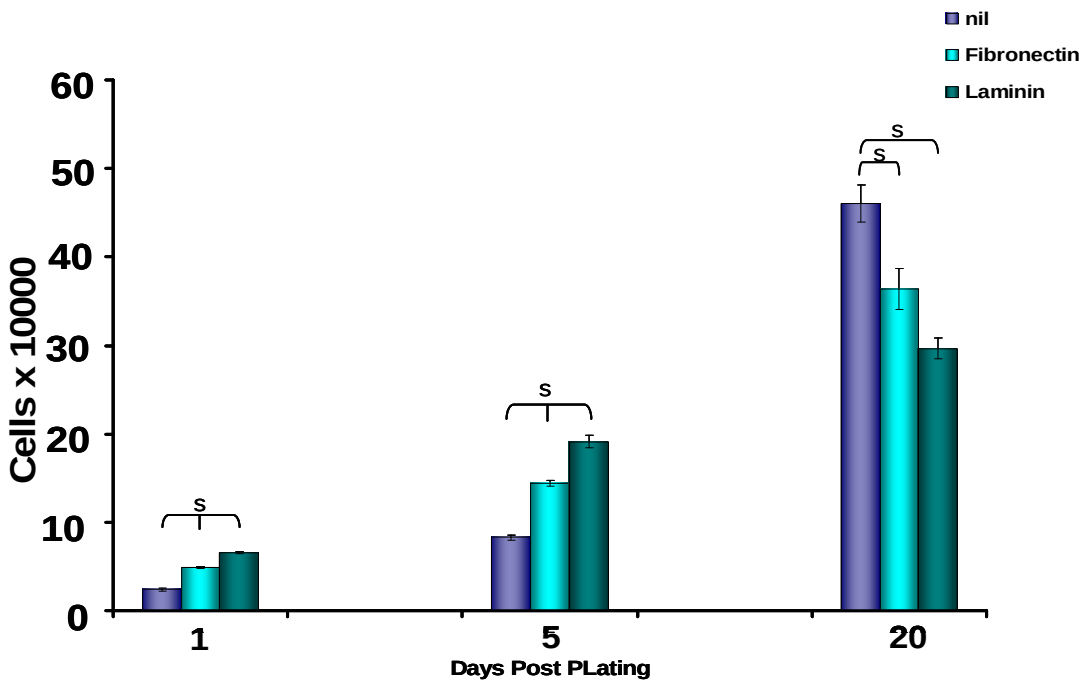


Figure 6.7: Quantitative effect of substrate coating of BioSorbPDX plates on C278F adhesion

Cells were seeded at 75,000/cm^2 in normal medium (replaced daily) onto untreated *BioSorbPDX* plates or plates coated with fibronectin or laminin. Cells were harvested and counted at days 1, 5 and 20. Data are expressed as the mean ± SEM for each group (n=3). Statistical differences among treatment groups were evaluated using analysis of variance (ANOVA) and the significance of differences was determined by *post hoc* testing using Bonferroni's method. **S** = significant ($p < 0.05$ was considered significant).

6.2.4: Alkaline phosphatase activity of C278F cells on treated *BioSorbPDX* plates.

Having observed and quantified the ability of cells carrying the C278F activating mutation to attach and expand on *BioSorbPDX* plates in the presence or absence of ECM coating, assessment of the cells ability to mineralise on the plates was undertaken.

Observed at day 20, ALP activity was greatest within the multilayered, nodular structures and appeared similar in cells growing on untreated membranes (**Fig. 6.8 B**) and those treated with fibronectin (**Fig. 6.8 D**) and laminin (**Fig. 6.8 F**). Interestingly, areas of cellular detachment were observed most obviously on plates treated with laminin,

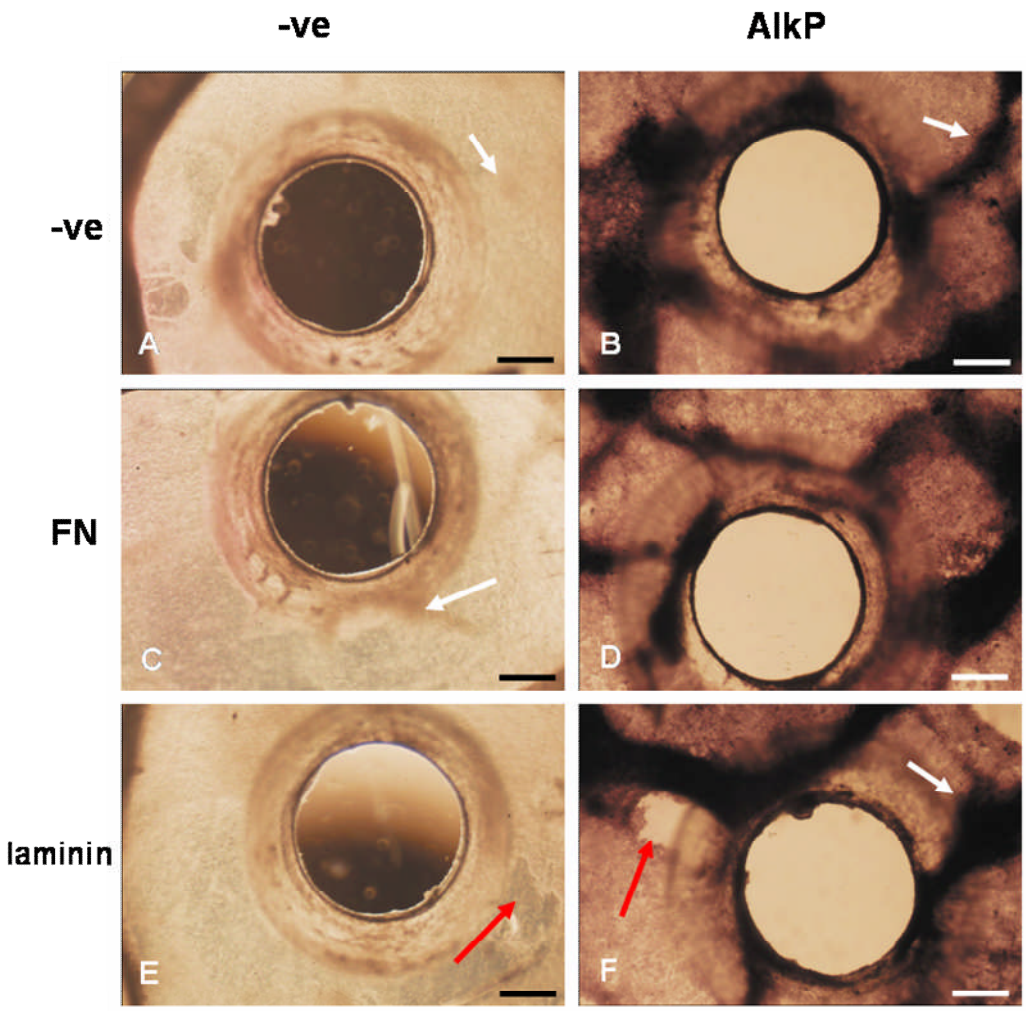


Figure 6.8: Alkaline phosphatase staining of C278F cells on treated *BioSorbPDX* plates. Day 20, C278F cells on *BioSorbPDX* plates in differentiating medium, demonstrated multilayered structures in unstained (**A,C,E**) and stained (**B,D,F**) wells. Staining for alkaline phosphatase was maximal in the nodular regions. White arrows indicate examples of multilayer, nodular structures. Areas of cellular detachment are indicated by red arrows. Cells were seeded at 75,000 cells/cm^2. Images obtained by plain backlit microscopy. Scale bars = 500 µm

6.2.5: Copolymer-bioactive glass composite membrane

This composite material consisted of bioactive glass fibres embedded onto an amorphous matrix of PLGA copolymer. The surface characteristics and microscopic appearance of the copolymer in this composite differed from the *BiosorbPDX*® studied previously in this chapter and did not allow the cells to be distinguished when viewed by microscopy (**Fig. 6.9 A**). Therefore, a vital cell tracker (CMFDA) was incorporated into the cells to allow observation under fluorescent microscopy (**Fig. 6.9 B**).

Initial observations of C278F cells 20 hours after seeding, revealed that attachment to both the co-polymer (**Fig. 6.9 A, B**) and bioactive glass fibre (**Fig. 6.9 C**) components of the composite was poor; they appeared rounded in shape with little evidence of development of pseudopodia or the typical elongated morphology of attached cells. Clumps of cells did however appear to be attracted towards the bioactive glass fibres (**Fig. 6.9 C**). Five days after seeding, cells were arranged longitudinally along the glass fibres suggesting that attachment had occurred (**Fig. 6.9 D**).

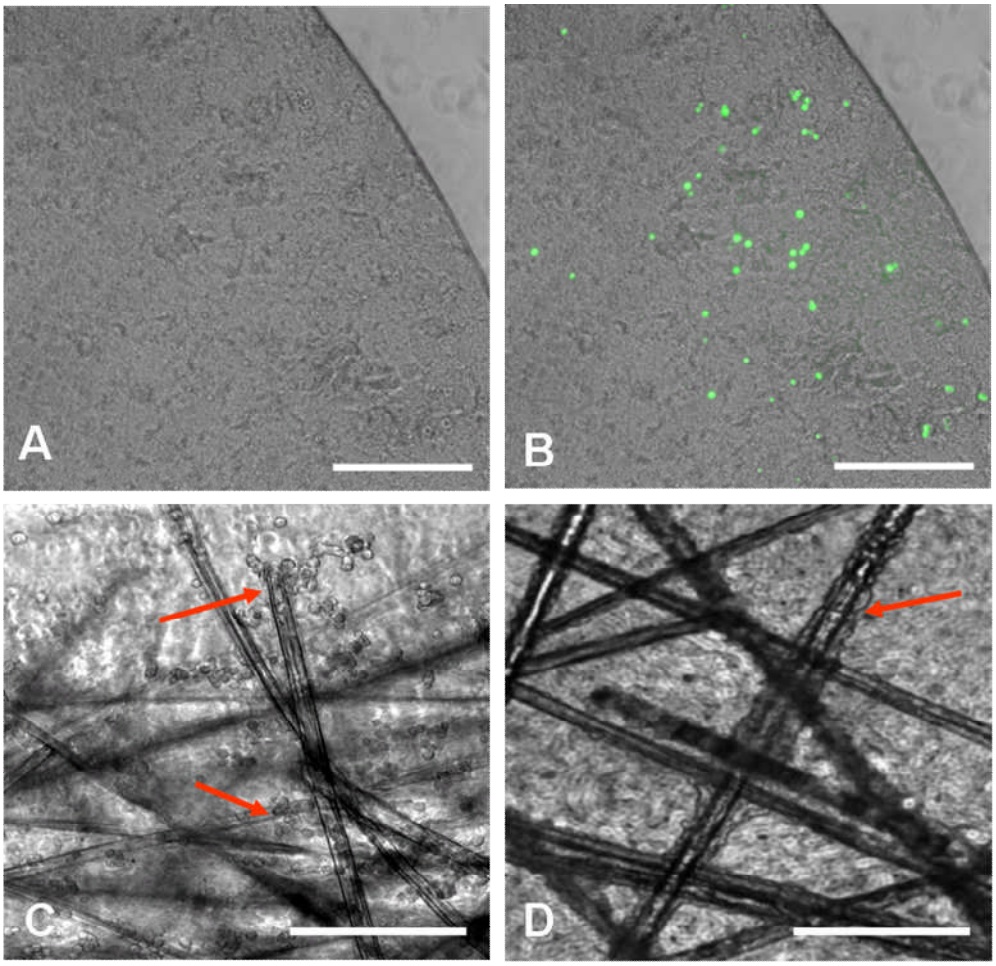


Figure. 6.9: Attachment of C278F cells onto copolymer-bioactive glass composite membrane
At 20 hours after seeding in normal medium, cells were difficult to observe on the copolymer component (**A**). Cells stained with CMFDA were visualised under fluorescent microscopy and the image digitally superimposed on the phase contrast image (**B**). When bioactive glass fibres were brought into focus, cells could be seen (arrow) clustering adjacent to the fibres (**C**). After 5 days, cells were attached (arrow) to the bioactive glass fibres (**D**). Cells seeded at 20,000 cells/cm^2. Scale bars = 200 µm (**A,B**) and 100 µm (**C,D**)
(See Appendix for note on published data)

6.3: Discussion

6.3.1: Effect of coating agents on cellular attachment

Initial observations revealed that the cell lines did not readily attach to the *BioSorbPDX* plate. This was an expected finding and consistent with the reports, using various cell types, of other authors who have demonstrated that polyester materials are only weakly hydrophilic and initially attach cells less efficiently than tissue culture plastic (Quirk et al., 2001; Moran et al., 2003; Lim et al., 2004). Furthermore, there are no cell recognition sites on the surfaces of PLGA scaffolds, leading to less than optimal cell affinity. It was therefore attempted to optimise cellular attachment to the *BioSorbPDX* plates as this appeared intuitively, to be a beneficial manipulation to enhance what would ultimately be an autograft, tissue engineering application. This study aimed to determine if there was a quantifiable benefit in coating potential scaffolds with ECM proteins and if there was any relative advantage between the ECM proteins.

Fibronectin and laminin coating of tissue culture plastic significantly enhanced the attachment of the cell lines which was demonstrated by the early morphological change in appearance of the WT and C278F cells. The visual impact of the enhanced attachment was obvious at early time points and appeared to have facilitated enhancement of cell numbers and growth at the later time points. This appearance was confirmed by quantification of the effect. Interestingly, at time points upto 4 hours, fibronectin and laminin similarly enhanced WT cell attachment with laminin having a significantly greater effect over fibronectin at 48 hours. However, attachment of cells carrying the mutation was enhanced to a greater degree by laminin, although at 48 hours this differential effect had been reversed to show fibronectin having the largest effect. The fact that laminin had a significantly greater affect on C278F cells soon after seeding, suggests that the mutation conferred on the cell an early selective receptor affinity for the different ECMs.

Whilst the RGD peptide is the single most important binding domain, other domains on the fibronectin and laminin molecules may be differentially bound by receptors expressed by the different cell lines. Moreover, passive adsorption could induce the competitive adsorption of other factors in the system and change the configuration of the adsorbed fibronectin or laminin molecules. As a result, its cell-binding activity would be influenced. For this reason it would have been informative to have also studied the effect of plates coated with the solitary RGD peptide.

It is more difficult to explain why there should have been switching between an early attachment advantage for laminin and a later advantage for fibronectin in the C278F cells. This effect is more likely to have been due to a cellular-ECM interaction than due to the chemico-physical interaction between the ECM protein and the culture plate surface, as laminin had produced increased attachment at 48 hours in the WT cells.

Culture dishes and *BioSorbPDX* plates were coated at the manufacturers recommended concentration and no attempt was made to study the concentration effect of laminin and fibronectin. However, it has been shown that attachment, proliferation and mineralisation of human osteoblasts is dose dependant for fibronectin or RGD peptide. (Yang et al., 2001; Behravesh et al., 2003).

Further studies of the cellular-ECM interaction would have been informative, both in quantifying the differential effect of extracellular proteins for enhancing attachment to scaffold materials and with respect to the differences in adhesion characteristics between the mutated and WT cells. These studies which could have involved such techniques as microcantilever (Bowen et al., 1997) micropipette (Athanassiou & Deligiannil 2001) or jet impingement (Gilberti et al., 2002) could have been linked with expression assays of cytoskeletal structures to facilitate the determination of the fundamental relationships between cell adhesion and function within these cells .

6.3.2: Attachment and growth on copolymer (*BioSorbPDX*) plates

Having demonstrated a clear attachment advantage for both fibronectin and laminin coated culture plastic, it was important to demonstrate if this advantage was transferable to the *BioSorbPDX* plates and whether this might influence mineralisation.

Fibronectin and laminin significantly increased C278F cell numbers on the *BioSorbPDX* plate at days 1 and 5. As with the study on tissue culture plastic, laminin had the greatest effect at the earlier time points. However at some point between day 5 and day 20 this advantage was lost, such that day 20 plates which were untreated, surprisingly had significantly greater cell numbers attached than those treated with either of the coating agents. This may have been due to the influence of the adsorbed proteins on cellular proliferation or apoptosis or both, as the cells moved from a proliferative to a differentiative phase. However, it is possible that the biomass of attached cells combined with even the minimalist agitation of the culture fluid when changing medium, may have caused shearing of cells from the plates and indeed voids can be seen on the plates where areas of cells have become detached (Figure 6.8). This would be consistent with the fact that polyesters lack chemical reactivity due to the absence of functional groups to support covalent

attachment. There may in fact come a point at which the mechanical attachment of cells to irregularities on the plate surface (see parallel grooves in Figure 6.1) is stronger than the surface-ECM interface, a concept supported by the findings of Matsuzaka et al., (1999).

The pattern of C278F growth was different on *BioSorbPDX* plates and culture dishes. Cells grown on *BioSorbPDX* appeared to reach confluence compared to their inability to reach full confluence (in the absence of growth factor) on tissue culture plastic. This might be explained by the recognition that nuclear shape which in turn may be influenced by overall cell shape has been shown to significantly affect cell function and regulatory pathways (Folkman & Mosconal 1978; Meyle et al., 1993; Lampin et al., 1997; Huang & Ingberl, 2000). This is also consistent with the finding that MC3T3-E1 differentiation and mineralisation is dependant upon the 3D conformation of the FN molecule which is itself dependant on the surface characteristics of the material upon which it is adsorbed (Stephansson et al., 2002).

A morphological feature which appears to be necessary across the spectrum of osteogenerative cells if functional bone is to be produced, is the development of mineralised nodules. Cellular growth on all of the plates in this study, supported the ability to form multilayered nodules. The nodules appeared to be more highly developed in the fibronectin coated plates, although this was not obvious when plates were stained for ALP expression. It should be noted however, that cells intended for ALP staining were grown in differentiating medium which may have influenced the growth and functionality of the cells.

6.3.3: Cellular alkaline phosphatase activity on *BioSorbPDX* plates

Successful mineralisation of matrix is one of the ultimate requirements for engineered bone tissues. It is recognised that potential materials for bone engineering are highly variable in their ability to support mineralisation (Calvert et al., 2005) and even the polylactic-polyglycolic (PLGA) co-polymer has been shown to greatly affect the ability of osteoblasts to mineralise matrix, depending upon the particular formulation of the material (Meredith et al., 2003). It was important therefore to establish whether the formulation being used in this study supported mineralisation.

The C278F cells at 20 days, stained for ALP in all conditions. The appearances suggested that staining was strongest on the plates not pre-coated. However this may have been due simply to more cells per unit area on the uncoated plates rather than an ability of these cells to express ALP more readily. There did not appear to be any obvious differential expression of ALP between the fibronectin and laminin coated plates.

The strong expression of ALP in all test conditions was interesting, in light of the finding in chapter 4, that C278F cells grown on tissue culture plastic did not express significant levels of ALP mRNA until day 30. While direct correlation of ALP mRNA expression and ALP protein activity cannot be made, Wang et al., (1999), have shown good correlation between mRNA expression and protein transcription for ALP in MC3T3-E1 cells. It can therefore be postulated that this apparent enhancement of ALP activity might be due to the influence of the co-polymer plate or its acidic breakdown products. Indeed this is supported by Ikarashi et al., (2000), who observed that poly-L-lactide (80% of the polyester in the *BiosorbPDX* plates) can stimulate ALP activity in MC3T3-E1 cells.

Attempts to stain for mineralisation using von Kossas' technique proved unsuccessful as the process caused almost total detachment of the cells from the plates.

6.3.4: Copolymer-Bioactive glass composite

A novel composite formulation of PLGA-bioactive glass became available towards the end of the project which allowed only limited investigation of the material.

Consistent with the previous findings of cellular growth on the *BiosorbPDX* plates, C278F cells did not attach readily to the amorphous co-polymer component of the material or the glass fibres. At 5 days however, cells were aligned longitudinally along the fibres.

Further work within our laboratory has demonstrated the ability of another formulation of this PLGA-bioactive glass composite to attract and allow proliferation and osteo-differentiation of the C278F line (Santos-Ruiz et al., 2007).

6.3.5: Summary

The findings in this chapter confirm that preosteoblasts carrying the C278F mutation attached poorly in the first instance to the co-polymer plates used in this study. It was shown that this early attachment can be enhanced by pre-coating with the extra-cellular matrix proteins, fibronectin and laminin and those cells carrying the mutation attach preferentially to laminin coated tissue culture plastic and co-polymer plates. Whilst early attachment is enhanced this seems to confer no later advantage in terms of cellular expansion. Indeed, when cells were in the late stages of osteodifferentiation there appeared to be a depletion of cell mass compared to untreated plates, which may have been due to detachment of cells at the material-ECM interface.

Co-polymer plates supported the formation of multilayered nodules and the expression of alkaline phosphatase, and may have demonstrated foci of mineralisation had the staining technique chosen not prevented the assay.

The limited analysis of cellular interaction with a novel composite material consisting of bioactive glass and co-polymer revealed that whilst early attachment of cells was poor, subsequent cellular attachment provides encouragement to proceed with further evaluation of this material.

Chapter 7: Final discussion

This study aimed to assess the feasibility of the MC3T3-E1 cell line carrying a craniosynostotic mutation, as a model for studying osteogenisis in craniosynostosis. It further sought to study the potential use of craniosynostotic osteoprogenitors in combination with bioresorbable materials and to identify potential modifiers to optimally manipulate these interactions.

Although transformed murine cells are inappropriate for therapeutic applications, MC3T3-E1 cells stably transfected with the human FGFR-C278F mutation coding for craniosynostosis, enabled studies on the growth and differentiation of craniosynostotic osteoprogenitor cells. These cells retained several properties of primary osteoblast cultures, including the ability to form a mineralised, collagenous, extracellular matrix and express gene products consistent with the osteoblast phenotype, thereby allowing the study of the functional effects of an FGFR2 mutation.

Cells carrying the FGFR2-C278F mutation appeared morphologically distinct from the MC3T3-E1 and WT cells. They required more frequent feeding and demonstrated higher levels of cell death suggesting a fundamental difference in phenotype and metabolic demands. When attached, they appeared more rounded implying lower levels of cellular prestress and possibly greater differentiation. Their attachment and clumping behaviour confirmed these differences although increased levels of Cx43 did not reach statistical significance. Hence, further studies directed at the mechanisms of cellular attachment in craniosynostotic cells, paying particular emphasis to the functional interaction between the extracellular matrix and the integrin linked cytoskeleton would be warranted.

The temporal expression of mRNA markers of osteodifferentiation did not wholly conform to expected findings. High early levels of osteocalcin in all cell lines and mineralisation of the C278F cells despite the delayed expression of alkaline phosphatase gives support to the findings of others that mineralisation can be dissociated from expression of osteocalcin and alkaline phosphatase (Beck et al., 1998; Ratisoontorn et al., 2003). This may also suggest that the well defined temporal relationships reported by the early workers with the MC3T3-E1 cell line, may have shifted due to significant clonal drift and subpopulation transformation, in keeping with the findings of Wang et al., (1999). In

this respect, the model studied could be questioned as to its suitability to represent and predict the effect of an *in vivo* mutation on progression of osteoprogenitors through the osteoblast lineage.

The study of growth factor manipulation and scaffold modification has been facilitated by these cells and should continue to allow the investigation and comparison between other mutations involved in pathological osteogenisis. This should allow further studies aimed at "reverting" the craniosynostotic phenotype without the need for genetic modification. In conclusion, the FGFR2-C278F cell line has been a useful osteoprogenitor model throughout this study and central to its findings. This model, compared with primary human craniosynostotic cultures for bio-scaffold engineering, has been further investigated in subsequent work carried out in our laboratory, (Santos-Ruiz et al., 2007).

The observation that FGF18 was expressed at higher levels in the early proliferative phase of C278F cell growth, suggested an important role for this growth factor in osteogenisis and in particular proliferative expansion. Therefore, for the first time the effect of exogenous FGF18 treatment on cells carrying a mutation for craniosynostosis was studied. In the presence of adequate serum concentrations, treatment with FGF18 enhanced proliferation of the cells in a dose dependant manner and allowed them to reach confluence, whereas C278F cells underwent cell death in low serum concentrations. This implied that these cells were more dependent on serum factors for cell survival and proliferation and as discussed in chapter 4, this may have been linked to levels of prestress within the cells and their dependence on adequate ECM attachments. Moreover, the levels of endogenous FGF18 produced by the cells were already raised and further treatment with exogenous mitogenic factors may have been additively detrimental when the cells were poorly attached. These findings suggest that FGF18, under the right conditions, may be a useful growth factor for the enhanced expansion of cell numbers.

Since the completion of this study the importance of FGF18 in osteogenisis has been further elucidated. Reinhold and Naski (2007), have shown that the transcription factors, *Runx2* and *TCF/Lef* which are transducers of *Wnt* signals and considered essential for osteogenisis, converge on the FGF18 promotor and suggest that expansion of an early osteoblast population through stimulation of cell proliferation will almost certainly be a key attribute of FGF18.

In further considering the possible clinical translation of FGF18 function, Haque et al., (2007), have recently found that chondrocytes within fracture callus of long bones undergoing distraction osteogenisis in a rabbit model, express FGF18 at high and persisting levels. It may be a significant observation that in children undergoing craniofacial distraction osteogenisis, the areas of bone which demonstrates regeneration are not those forming in membranous bone, but those at the skull base formed by endochondral ossification (Fig. 1.5B).

Taken together, these findings and observations suggest that FGF18 is a worthy candidate for further study in future developments of osteogenisis and bone engineering.

There are many different interacting and complex factors which will ultimately affect the progress of osteogenesis on bio-composite scaffolds. It has been shown that osteoprogenitor cells carrying this particular FGFR2-C278F mutation, attach, grow and express an osteoblast like phenotype on PLGA (*Biosorb PDX*) fixation plates and that the ECM molecules, fibronectin and laminin, support early attachment and growth of these cells. However, a non-activating FGFR mutation such as those found in Aperts syndrome may respond differently to the same proteins. Likewise, the optimum concentration of growth factors for any particular culture system on any particular scaffold formulation or architecture may be very different. This study has hence demonstrated that these complexities overwhelm the conventional “one-sample/one-measurement” approach to the evaluation of bioactive systems.

In recognition of these multifactorial and complex interactions, superior bioactive systems must be designed by taking into account the synergisms of the most important of these factors. To this end, attempts are being made to approach this problem by the development of combinatorial libraries using such techniques as micro-array gene analysis, chromatographic gradient libraries and high-throughput polymer concentration and surface libraries (Chicurel, 2001; Meredith et al., 2003). Ultimately, this approach might lead to the customised engineering of bone for any individual patient, dependant upon the genotype/phenotype of the autologous osteoprogenitors

Overall, this study has found the transfected MC3T3-E1 cell lines to be a useful model for studying the molecular basis of craniosynostoses and the response of mutated osteoprogenitors to different materials, extracellular matrix molecules and growth factors. Fibronectin and laminin supported cellular attachment and growth to facilitate the early expansion of mutated osteoprogenitors on a commercially available bioresorbable

scaffold. FGF18 expression and its exogenous effects on osteoprogenitor behaviour, suggest that this is an important growth factor in osteogenisis. This study provides the basis for further investigation aimed at using these molecules to develop bio-composites for autologous cell therapy, without the medical and ethical hazards of genetic manipulation, in the management of these challenging surgical reconstructions.

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Appendix :

Publication Note

The contribution of the author to the referenced publication Santos-Ruiz et al., (2007), involved reviews and revisions of the original manuscript and contribution of the following data:

p202, Figure 1, Osteocalcin expression data
p203, Figure 2 (G,H,I), MC3T3/WT/C278F von Kossa staining at 21 days
p208 Figure 9 (A,B,C), MC3T3 on culture wells, BiosorbPDX with and without alizarin

The copolymer-bioglass membrane which appears in Figure 6.9 of this thesis was material which became available towards the end of this study and is an earlier formulation than that which appears in the published paper.

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